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(54) Recombinant coccidiosis vaccines.

(57) This invention provides DNA sequences coding for Eimeria surface antigens, recombinant vectors containing such DNA sequences, transformed host organisms containing such vectors, and methods for producing the antigens using the transformed microorganisms. Methods are also provided for protecting poultry against coccidiosis using the Eimeria surface antigens. The surface antigens can be administered for such protection either as purified proteins or in the form of DNA encoding the proteins in a suitable viral vector such as vaccinia virus.

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Recombinant coccidiosis vaccines

Coccidiosis is a disease of poultry caused by intracellular protozoan parasites of the genus Eimeria. The disease is endemic in large, intensive poultry breeding establishments and the estimated cost of control of the disease through chemotherapy exceeds $100 million each year in the United States of America alone. The development of resistance to the anti-coccidial drugs necessitates a continuing development of new agents, at a time when drug development is becoming increasingly expensive and consumer acceptance of drug residues in food animals is diminishing.

Protective immunity to natural coccidiosis infection has been well documented. Controlled, daily administration of small numbers of viable oocysts for several weeks has been shown to result in complete immunity to a challenge infection of a normally virulent dose [Rose et al., Parasitology 73:25 (1976); Rose et al., Parasitology 88:199 (1984)]. The demonstration of acquired resistance to infection suggests the possibility of constructing a vaccine to induce immunity in young chickens, circumventing the need for chemical coccidiostats. In fact, such a concept has been tested in the Coccivac® formulation of Sterwin Laboratories, Opelika, Alabama, U.S.A.

With a view to producing a coccidiosis vaccine, Murray et al., European Patent Application, Publication No. 167.443, prepared extracts from sporozoites or sporulated oocysts of Eimeria tenella which contain at least 15 polypeptides, many of which were associated with the surface of the sporozoite. Injection of these extracts into chickens reduced cecal lesions following oral inoculation with virulent E. tenella sporulated oocysts. More recently, Schenkel et al., U.S. Patent No. 4,650,676, disclosed the production of monoclonal antibodies against E. tenella merozoites. Using these antibodies, Schenkel et al. identified a number of antigens against which the antibodies were directed. By pre-incubating E. tenella sporozoites with these antibodies and then introducing the treated sporozoites into the ceca of chickens, Schenkel et al. were able to show some reduction in cecal lesion scores, compared to untreated sporozoite controls.

Advances in recombinant DNA technology have made another approach available, viz. subunit vaccines. In the application of current recombinant DNA procedures, specific DNA sequences are inserted into an appropriate DNA vehicle, or vector, to form recombinant DNA molecules that can replicate in host cells. Circular double-stranded DNA molecules called plasmids are frequently used as vectors, and the preparation of such recombinant DNA forms entails the use of restriction endonuclease enzymes that can cleave DNA at specific base sequence sites. Once cuts have been made by a restriction enzyme in a plasmid and in the segment of foreign DNA that is to be inserted, the two DNA molecules may be covalently linked by an enzyme known as a ligase. General methods for the preparation of such recombinant DNA molecules have been described by Cohen et al. [U.S. Patent No. 4,237,224], Collins et al. [U.S. Patent No. 4,304,863] and Maniatis et al. [Molecular Cloning: A Laboratory Manual, 1982, Cold Spring Harbor Laboratory]. Because they illustrate much of the state of the art, these references are hereby incorporated by reference.

Once prepared, recombinant DNA molecules can be used to produce the product specified by the inserted gene sequence only if a number of conditions are met. Foremost is the requirement that the recombinant molecule be compatible with, and thus capable of autonomous replication in the host cell. Much recent work has utilized Escherichia coli as a host organism, because it is compatible with a wide range of recombinant plasmids. Depending upon the vector/host cell system used, the recombinant DNA molecule is introduced into the host by transformation, transduction or transfection.

Detection of the presence of recombinant plasmids in host cells may be conveniently achieved through the use of plasmid marker activities, such as antibiotic resistance. Thus, a host bearing a plasmid coding for the production of an ampicillin-degrading enzyme could be selected from unaltered cells by growing the host in a medium containing ampicillin. Further advantage may be taken of antibiotic resistance markers where a plasmid codes for a second antibiotic-degrading activity at a site where the selected restriction endonuclease makes its cut and the foreign gene sequence is inserted. Host cells containing properly recombinant plasmids will then be characterized by resistance to the first antibiotic but sensitivity to the second.

The mere insertion of a recombinant plasmid into a host cell and the isolation of the modified host will not in itself assure that significant amounts of the desired gene product will be produced. For this to occur, the foreign gene sequence must be fused in proper relationship to a signal region in the plasmid for DNA transcription called a promoter. Alternatively, the foreign DNA may carry its own promoter, as long as it is recognized by the host. Whatever its origin, the promoter is a DNA sequence that directs the binding of RNA polymerase and therefore "promotes" the transcription of DNA to messenger RNA (mRNA).

Given strong promotion that can provide large quantities of mRNA, the ultimate production of the desired gene product will be dependent upon the effectiveness of translation from mRNA to protein. This, in

turn, is dependent upon the efficiency of ribosomal binding to the mRNA. In E. coli, the ribosome-binding site on mRNA includes an initiation codon (AUG) and an upstream Shine-Dalgarno (SD) sequence. This sequence, containing 3-9 nucleotides and located 3-11 nucleotides from the AUG codon, is complementary to the 3′ end of E. coli 16S ribosomal RNA (rRNA) [Shine and Dalgarno, Nature 254:34 (1975)]. Apparently, ribosomal binding to, mRNA is facilitated by base pairing between the SD sequence in the mRNA and the sequence at the 16S rRNA 3′ end. For a review on maximizing gene expression, see Roberts and Lauer, Methods in Enzymology 68:473 (1979).

An alternative expression system has been developed based on the lacz operon in combination with lambda phage vectors (Huynh et al., in DNA Cloning: Volume I, D.M. Glover, Ed.). In this system, the structural gene for β-galactosidase along with the inducible promoter controlling its expression have been engineered into the phage vector. A unique cloning site at the 3′ end of the gene for β-galactosidase results in a gene fusion upon the insertion of a cDNA copy of an mRNA or a genomic DNA fragment containing a protein-coding region.

Expression of the β-galactosidase gene results in the production of a fusion protein containing 114 kd of β-galactosidase and a carboxy terminal polypeptide encoded by the cDNA insert, provided that the insert contains an open reading frame in the same register as the reading frame for β-galactosidase. A phage containing a gene whose product is recognized by a monoclonal or a polyclonal antiserum can thus be identified by immunologic screening of the library following induction of expression of the fusion protein using β-D-thiogalactopyranoside (IPTG) to inactivate the lacZ repressor. This expression vector system combines the efficiency of the phage system in packaging DNA and introducing it into E. coli cells with an increased stability of polypeptide fusions with β-galactosidase.

In the vaccine subunit approach, a subunit of the whole infectious organism is delivered to the host animal in an immunologically relevant context. The subunit might be a protein purified from the parasite, a recombinant protein or protein fragment expressed in a heterologous system, a synthetic peptide comprising a single neutralizing determinant or a protein introduced by a viral vector such as vaccinia. The host immune system mounts a specific response to the subunit without ever being exposed to the whole parasite. Upon challenge with a virulent dose of the infectious organism, the host immune system mounts a successful defense, instructed only by the vaccine subunit to which it had been previously exposed.

Evidence can be found in the literature for the involvement of circulating antibodies, secretory IgA in the intestinal epithelium [Davis et al., Immunology 34:879 (1978)], and the cell-mediated immune system [Giambroni et al., Poultry Science 59:38 (1980)] in acquired resistance to coccidiosis. For a review, see P.S. Davis in Avian Immunology, M.E. Rose, Ed., British Poultry Science, Ltd., Edenberg, pp. 361-385 (1981). The probable involvement of various arms of the immune system means that complete and lasting protection may necessitate the ability to mimic specific aspects of the natural infectious process. These aspects include local exposure at the site where protection is desired, evocation of an inflammatory response to marshall antigen processing cells, presentation of an appropriate parasite antigen and possibly association with MHC determinants in a particular membrane configuration.

This invention provides purified proteins or fragments thereof having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen.

More particularly, this invention provides proteins having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen, which surface antigen has an apparent molecular weight of about 28, 37, 120 or greater than 200 kilodaltons and specifically binds to one or more monoclonal antibodies deposited with the American Type Culture Collection (ATCC) and assigned accession Nos. HB 9707 through HB 9712. Examples for said proteins are proteins having the amino acid sequences shown in Fig. 15, Fig. 17, Fig. 19 and Fig. 21 and its functional equivalents. Said functional equivalents are proteins having an amino acid sequence derived from the amino acid sequences mentioned above by additions, deletions, insertion and amino acid substitutions, provided that these proteins retain one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen. The said proteins may be used for the immunization of poultry against coccidiosis.

This invention further provides antibodies directed against the above-mentioned proteins especially monoclonal antibodies such as the monoclonal antibodies with the accession Nos. HB 9707, HB 9708, HB 9709, HB 9710, HB 9711 and HB 9712.

This invention still further provides DNA sequences encoding the above-mentioned proteins, recombinant vectors comprising such DNA sequences especially recombinant vectors which are capable of directing the expression of the said DNA sequences in compatible host organisms and host organisms transformed with such recombinant vectors, especially transformed host organisms which are capable of expressing the DNA sequences encoding an above-mentioned protein comprised in the said recombinant vector.

This invention still further provides a process for the preparation of a protein having one or more immunoreactive and/or antigenic determinants of an Eimeria tenella surface antigen, which process comprises:

(a) culturing a host organism transformed with a recombinant vector comprising a DNA sequence encoding said protein under conditions in which the DNA sequence is expressed; and

(b) isolating the protein or fragment from the culture.

This invention still further provides a process for the preparation of an above-mentioned transformed host organism which process comprises transforming a host organism with a recombinant vector comprising a DNA sequence encoding a protein of the present invention using methods known per se.

This invention still further provides vaccines for protecting poultry against coccidiosis comprising one or more of the proteins of the invention and a physiologically acceptable carrier.

This invention still further provides vaccines for protecting poultry against coccidiosis comprising a viral vector containing a DNA sequence or fragment thereof coding for a protein of the invention, which viral vector is capable of expressing the DNA sequence or fragment, and a physiologically acceptable carrier.

This invention still further provides a method for protecting poultry against coccidiosis, which method comprises administering an effective amount of a vaccine of the invention to a young fowl which is susceptible to coccidiosis.

The present invention can be more readily understood by reference to the following description of the invention and the Example in connection with the following Figures in which:

Fig. 1 shows the results of an E. tenella sporozoite ELISA. Dilutions of immune mouse serum (MS 107-2;Δ) and control mouse serum (X) were incubated with 4 X 10^4 live purified sporozoites. Specific antibody bound to the sporozoites was detected with a peroxidase-conjugated anti-mouse IgG antibody and the peroxidase substrate o-phenylenediamine. The OD_{492nm} was read in a Titertek Multiscan® plate reader.

Fig. 2 shows the results of a Western blot assay performed with proteins solubilized from E. tenella sporozoites. Solubilized sporozoite proteins were separated by reducing SDS-polyacrylamide gel electrophoresis in 12.5% gels, transferred to nitrocellulose membranes and reacted with each antibody. The specific proteins recognized by each antibody were visualized with a peroxidase-conjugated anti-mouse IgG antibody and the peroxidase substrate 4-chloro-1-naphthol. The antibody which was reacted with each strip is noted at the top of the strip.

Fig. 3 shows the results of a Western blot assay performed with proteins solubilized from sporozoites and merozoites of E. tenella, and from sporozoites of E. acervulina. Various monoclonal antibodies and sera were incubated with nitrocellulose bound Eimeria proteins and visualized as explained in the description of Fig. 2. The monoclonal antibodies used included 3A5 (1), 20C6 (2), 7D1 (3), 13A6 (4), 6A5 (5) and a control antibody that was unreactive to the Eimeria proteins. The sera used included mouse No. 107-2 immune serum (7) and control serum (8).

Fig. 4 shows in the left panel the results of an immunoprecipitation assay with ^{125}I-labeled surface proteins of E. tenella sporozoites. Sporozoite surface proteins were labeled by either the IODOGEN or IODOBEADS method, solubilized and visualized following SDS-polyacrylamide gel electrophoresis in 12.5% gels by autoradiography. The right panel shows the results of immunoprecipitation of ^{125}I-labeled sporozoite surface proteins by serum from mice immunized with live sporozoites. Immune mouse sera (105-1, 105-2, 105-3, 107-1, 107-2 and 107-3) and control mouse serum (Control) were incubated with ^{125}I-sporozoite surface proteins, and the immune complexes were captured by an anti-mouse antibody coupled to agarose. The immune complexes were solubilized with Laemmli sample buffer, separated by SDS-gel electrophoresis in 12.5% gels and visualized by autoradiography. M represents the molecular weights of standard marker proteins in kilodaltons.

Fig. 5 shows the results of immunoprecipitation of ^{125}I-sporozoite surface proteins by monoclonal antibodies. The procedure for identifying the ^{125}I-proteins bound by each antibody is explained in the description of Fig. 4. Specific sporozoite monoclonal antibodies used and control antibody (control) are indicated at the top of each gel lane. Molecular weights of standard marker proteins are shown in kilodaltons.

Fig. 6 shows phase contrast micrographs and immunofluorescence staining pattern micrographs using various monoclonal antibodies, of air-dried E. tenella sporozoite slide preparations. The left sides of panels A, B, C and D are phase contrast micrographs showing intact elongated sporozoites with a large posterior refractile body (PRB), a small anterior refractile body (ARB) and the apical end (A) opposite the posterior refractile body. The right sides of panels A, B, C and D show slides which were treated with monoclonal antibodies 14C3 (specific for surface antigens), 6A5 (specific for surface and refractile body

protein), 11D2 (specific for sporozoite apical tip) and control antibody, respectively. The antibodies bound to the preparations were localized with rhodamine-conjugated anti-mouse antibodies, visualized by epi-fluorescence using a Leitz Dialux 22® microscope. All micrographs are 630X.

Fig. 7 shows antibody staining of intracellular sporozoites and the developing parasite in chicken kidney cells. Chicken kidney cells were infected with sporozoites, and at the indicated times after infection the cells were processed for antibody staining. The cultures were washed before fixation to remove any extracellular sporozoites. Phase contrast and corresponding immunofluorescence micrographs were made using antibodies 7D4, 8A2, 7B2 and 15A3 as indicated. The antibodies bound to the preparations were localized with rhodamine-conjugated anti-mouse antibodies, visualized by epifluorescence. All micrographs are 630X.

Fig. 8 shows antibody staining of intracellular sporozoites and the developing parasite in chicken kidney cells. Phase contrast micrographs and corresponding immunofluorescence micrographs were made using monoclonal antibodies 14B1 and 19D6, immune chick sera and fluorescent second antibodies, at the indicated times.

Fig. 9 shows the neutralization of intracellular sporozoite development by anti-sporozoite antibodies. Purified E. tenella sporozoites were preincubated for 1 hour at 40° C with either control antibody (X) or anti-sporozoite antibodies 7D4 (□), 8A2 (○), 14B1 (●) or 6A5 (■) and then allowed to infect MDBK cell cultures. Sporozoites were also preincubated with media (Δ) or with the anti-coccidial drug, lasalocid (*).

After infection, the development of the intracellular sporozoite was measured by the incorporation of ^{3}H-uracil into the cell cultures. Since lasalocid prevents intracellular development of the sporozoite, cultures pretreated with this drug showed minimal incorporation of ^{3}H-uracil.

Fig. 10 shows the results of SDS-polyacrylamide gel electrophoretic/Western Blot analysis of 65 Kd-β-galactosidase fusion protein samples or other samples as noted. The Western Blot analysis was carried out using murine anti-β-galactosidase antibody (panel A) or pooled monoclonal antibodies 7D1, 7D4 and 20C6 (panel B) in conjunction with goat anti-mouse HPOD conjugate. The lanes in both panels represent (1) β-galactosidase, (m) prestained molecular weight markers, the sizes of which are indicated to the left of plate A in kd, (2) total cell pellet protein, (3) protein released from the cell pellet by sonication and (4) protein solubilized by guanidine-HCl from the pellet after sonication.

Fig. 11 is a schematic representation of plasmid pEV/2-4, a 65 kd protein expression plasmid containing a 1.7 kb EcoRI DNA insert from phage λm2-4. Positions of various restriction enzyme sites in the insert are shown relative to the EcoRI site, including PstI (P, at bp 53 and 776), KpnI (K, at bp 202), BstNI (B, at bp 584, 1303 and 1412) and Sau3A (S, at bp 1017 and 1439).

Fig. 12 is a map of pEV3-SEQ, containing a polylinker with the indicated sites inserted between the EcoRI and SalI sites of pEV-vrf3. The synthetic oligonucleotide CGGTCGACTCGAGCCA, indicated by the dashed arrow, was used as a primer for chain-termination DNA sequence analysis.

Fig. 13 is a restriction map of cDNA clones encoding proteins recognized by monoclonal antibody 6A5. Restriction endonuclease sites used for Maxam-Gilbert DNA sequence analysis of the 1.1 kb cDNA are shown. The EcoRI site in parentheses is at the end of the 0.9 kb cDNA. The bar above the map shows the open reading frame predicted from the DNA sequence, with the potential signal peptide filled in. The lines below the map indicate the exoIII deletions used for chain-termination sequence analysis.

Fig. 14 is the nucleotide sequence of the 1.1 kb cDNA molecule encoding the 20 kd protein recognized by monoclonal antibody 6A5.

Fig. 15 is the amino acid sequence of the protein of Fig. 14, predicted from the nucleotide sequence of that figure.

Fig. 16 is the nucleotide sequence of the 1.7 kb cDNA molecule encoding the 65 kd protein recognized by monoclonal antibodies 7D1, 7D4 and 20C6.

Fig. 17 is the amino acid sequence of the protein of Fig. 16, predicted from the nucleotide sequence of that figure and confirmed by sequence analysis of tryptic peptides produced from the expressed 65 kd protein. Regions in the overall amino acid sequence corresponding to some of these peptides are shown underlined. The determined sequences of these peptides are overlined.

Fig. 18 is the nucleotide sequence of the 1.1 kb cDNA molecule encoding the 28 kd protein recognized by monoclonal antibody 8A2.

Fig. 19 is the amino acid sequence of the protein of Fig. 18, predicted from the nucleotide sequence of that figure.

Fig. 20 is the nucleotide sequence of the 3.2 kb cDNA molecule encoding the protein recognized by monoclonal antibody 7B2.

Fig. 21 is the amino acid sequence of the protein of Fig. 20, predicted from the nucleotide sequence of that figure.

Fig. 22 is an SDS polyacrylamide gel electrophoretic analysis of the immunoaffinity-purified 65 kd protein. The gel was visualized by Coomassie blue stain and by Western blot analysis. Lanes 2 and 4 and 3 and 5 contain the purified protein from two preparations. Lanes 1 and 6 contain a mixture of molecular weight marker proteins having the molecular weights shown to the left and right of the figure.

Fig. 23 is an HPLC elution profile of a β-mercaptoethanol reduced (panel A) and unreduced (panel B) tryptic digest of the 65 kd protein, showing absorbance at 215 mμ as a function of column retention time.

Fig. 24 shows restriction maps of four elements of the basic vector used for recombination of genes coding for coccidial antigens into vaccinia virus. These elements include the 7.5K promoter element (a and b, left), the TK locus (a and b, right), part of plasmid pUC8 (c) and the polycloning site from Ml3tgl3l (d). The direction of transcription of the viral 7.5K and TK promoters is from left to right (i.e., from the BglII to the EcoRI restriction site in the polylinker.

Fig. 25 shows the amino acid sequence of the N-terminus of the Eimeria antigen recognized by monoclonal antibody 8A2 (A) expressed from a construct containing the AUG translation start codon in the polylinker element of the vector of Fig. 24, and (B) fused to the malarial 190 kd leader segment (first 34 amino acids) and the polylinker of the cloning vector of Fig. 24 (next 13 amino acids). During the maturation process of the protein, the first 19 amino acids at the N-terminus may be cleaved at the position indicated by a colon.

In the figures, standard single letter abbreviations are used to represent nucleotides, and standard one or three letter abbreviations are used to represent amino acids. The meanings of these abbreviations can be found in standard biochemistry textbooks, such as Lehninger, Principles of Biochemistry, 1984, Worth Publishers, Inc., New York, pp. 96, 798.

As used herein, the following terms shall have the following meanings:

"20 kd protein" means a recombinant or synthetic protein having an apparent molecular weight of about 20 kilodaltons in SDS polyacrylamide gel electrophoresis which binds specifically to monoclonal antibody 6A5. This antibody also specifically reacts with an Eimeria surface antigen (from a whole extract of Eimeria proteins) having an apparent molecular weight of about 28 kilodaltons in SDS gels. This antigen is present in the sporozoite developmental stage. The nucleotide sequence of a cDNA molecule encoding this protein and the amino acid sequence predicted therefrom are shown in Figs. 14 and 15, respectively.

"65 kd protein" means a recombinant or synthetic protein having an apparent molecular weight of about 65 kilodaltons in SDS polyacrylamide gel electrophoresis which binds specifically to monoclonal antibodies 7D1, 7D4 and 20C6. These antibodies also specifically react with a surface antigen from Eimeria extracts having an apparent molecular weight of about 120 kilodaltons in SDS gels. This antigen is present in the sporozoite, schizont and merozoite developmental stages. The nucleotide sequence of a cDNA molecule encoding this protein and the amino acid sequence predicted therefrom are shown in Figs. 16 and 17, respectively.

"28 kd protein" means a recombinant or synthetic protein having an apparent molecular weight of about 28 kilodaltons in SDS polyacrylamide gel electrophoresis which binds specifically to monoclonal antibody 8A2. This antibody also specifically reacts with an Eimeria surface antigen having an apparent molecular weight of about 37 kilodaltons in SDS gels. This antigen is present in the sporozoite, schizont and merozoite developmental stages. The nucleotide sequence of a cDNA molecule encoding this protein and the amino acid sequence predicted therefrom are shown in Figs. 18 and 19, respectively.

"45 kd protein" means a recombinant or synthetic protein having an apparent molecular weight of about 45 kilodaltons in SDS polyacrylamide gel electrophoresis which binds specifically to monoclonal antibody 7B2. This antibody also specifically reacts with an Eimeria surface antigen having an apparent molecular weight of greater than 200 kilodaltons in SDS gels. This antigen is present in the sporozoite developmental stage. The nucleotide sequence of a cDNA molecule encoding this protein and the amino acid sequence predicted therefrom are shown in Figs. 20 and 21, respectively.

The term "protein having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen" means a protein having one or more regions or epitopes which are capable of eliciting an immune response in an immunologically competent host organism and/or are capable of specifically binding to a complementary antibody.

Because of the degeneracy of the genetic code, it will be understood that there are many potential nucleotide sequences (functional equivalents) that could code for the amino acid sequences shown in Figs. 15, 17, 19 and 21. It should also be understood that the nucleotide sequences of the DNA sequences and fragments of the invention inserted into vectors may include nucleotides which are not part of the actual

structural genes, as long as the recombinant vectors containing such sequences and fragments are capable of directing the production in an appropriate host organism of a protein or fragment having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen.

Moreover, amino acid substitutions in proteins which do not essentially alter biological and immunological activities have been known to occur and have been described, e.g., by Neurath et al. in "The Proteins", Academic Press, New York (1979), in particular in Fig. 6 at page 14. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, and vice versa.

Such functionally equivalent nucleotide sequence variations and amino acid substitutions of the exemplary embodiments of this invention are within the scope of the invention as long as the resulting proteins retain one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen.

The term "fragment" means a DNA sequence or protein comprising a subsequence of one of the cDNA'S or proteins of the invention. Such fragments can be produced by enzymatic cleavage of the larger molecules, using restriction endonucleases for the DNA and proteases for the proteins. The fragments of the invention, however, are not limited to the products of any form of enzymatic cleavage but include subsequences, the termini of which do not correspond to any enzymatic cleavage points. Such fragments can be made, e.g., by chemical synthesis, using the sequence data provided herein. DNA fragments can be produced by incomplete complementary DNA (cDNA) synthesis from isolated messenger RNA (mRNA). Protein fragments can also be produced by expressing DNA fragments encoding the protein fragments. Such protein fragments can be useful in this invention if they contain a sufficient number of amino acid residues to constitute an immunoreactive and/or antigenic determinant. Generally, at least about 7 or 8 residues are needed. As explained below, it may be necessary to couple such fragments to an immunogenic carrier molecule, to make them immunoreactive.

The proteins of this invention can be made my methods known in the art such as by recombinant DNA methodology, chemical synthesis or isolation from Eimeria preparations.

DNA needed to make the proteins of this invention could be chemically synthesized, using the nucleotide sequence information provided in Figs. 14, 16, 18 and 20. Such chemical synthesis could be carried out using any of the known methods, although the phosphoramidite solid support method of Matteucci et al. [J. Am. Chem. Soc. 103:3185 (1981)] is preferred.

Alternatively, cDNA can be made from Eimeria mRNA. Messenger RNA can be isolated from Eimeria sporulating oocysts or merozoites using standard techniques. These mRNA samples can then be used to produce double-stranded cDNA as described by Maniatis et al., supra. This cDNA can then be inserted into an appropriate cloning vector which can be used to transform E. coli, to produce a cDNA library.

The cDNA library can then be screened using the cloned genes of this invention, or fragments thereof, as probes. Such genes or fragments can be radiolabeled, e.g., by nick-translation using Pol I DNA polymerase in the presence of the four deoxyribonucleotides, one of which contains ^{32}P in the α position (Maniatis et al., supra, p. 109), for use as probes.

Although Eimeria tenella was used as an mRNA source in the Examples below, the cloned genes from this species can be used as probes to isolate genes from other species of Eimeria, due to DNA sequence homology among the various species.

Once identified and isolated, the Eimeria genes of this invention are inserted into an appropriate expression vehicle which contains the elements necessary for transcription and translation of the inserted gene sequences. Useful cloning vehicles may consist of segments of chromosomal, nonchromosomal and synthetic DNA sequences such as various known bacterial plasmids, phage DNA, combinations of plasmids and phage DNA such as plasmids which have been modified to employ phage DNA or other expression control sequences, or yeast plasmids. Specific cloning vehicles which could be used and are known to the man skilled in the art include but are not limited to the pEV-vrf plasmids (pEV-vrf1, -2 and -3); SV40; adenovirus; yeast; lambda gt-WES-lambda B; Charon 4A and 28; lambda-gt-11-lambda B; M13-derived vectors such as pUC8, 9, 18 and 19, pBR313, 322 and 325; pAC105; pVA51; pACY177; pKH47; pACYC184; pUB110; pMB9; co1E1; pSC101; pm121; RSF2124; pCR1 or RP4.

The insertion of the Eimeria genes into a cloning vector is easily accomplished when both the genes and the desired cloning vehicle have been cut with the same restriction enzyme or enzymes, since complementary DNA termini are thereby produced. If this cannot be accomplished, it may be necessary to modify the cut ends that are produced by digesting back single-stranded DNA to produce blunt ends, or by achieving the same result by filling in the single-stranded termini with an appropriate DNA polymerase. In this way, blunt-end ligation with an enzyme such as T4 DNA ligase may be carried out. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini. Such linkers may comprise specific oligonucleotide sequences that encode restriction site recognition sequences. The

cleaved vector and the Eimeria genes may also be modified by homopolymeric tailing, as described by Morrow [Methods in Enzymology 68:3 (1979)].

Many of the cloning vehicles that may be used in this invention contain one or more marker activities that may be used to select for desired transformants, such as ampicillin and tetracycline resistance in pBR322, ampicillin resistance and β-galactosidase activity in pUC8, and ampicillin resistance in pEV-vrf2. Selection of host cells into which such vectors have been inserted is greatly simplified when the host cells otherwise lack the activities contributed by the vectors.

It should be understood that the nucleotide sequences of the Eimeria genes inserted at a selected site in a cloning vehicle may include nucleotides which are not part of the actual structural genes. Alternatively, the gene may contain only part of the complete wild-type gene. All that is required is that the gene fragments inserted into the cloning vehicle be capable of directing the production in an appropriate host organism of a polypeptide or protein having at least one immunoreactive and/or antigenic determinant of an Eimeria surface antigen.

The selection of an appropriate host organism is affected by a number of factors known in the art. These factors include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. A balance of these factors must be struck, and it must be understood that not all hosts will be equally effective for expression of a particular recombinant DNA molecule.

Suitable host unicellular organisms which can be used in this invention include but are not limited to plant, mammalian or yeast cells and bacteria such as Escherichia coli, Bacillus subtilis, Bacillus stearothermophilus and Actinomyces. Especially preferred is Escherichia coli strain MC1061, which has been described by Casadaban et al. [J. Mol. Biol. 138:179 (1980)]. This strain can be used, or any other strain of E. coli K-12 containing the plasmid pRK248clts. Plasmid pRK248clts for use in other E. coli K-12 strains is available from the American Type Culture Collection and has accession No. ATCC 33766. E. coli strain MC1061 has also been deposited and has accession No. ATCC 53338.

Transfer of the recombinant cloning vector into the host cell may be carried out in a variety of ways. Depending upon the particular vector/host cell system chosen, such transfer may be effected by transformation, transduction or transfection. Once such a modified host cell is produced, the cell can be cultured and the protein expression product may be isolated from the culture.

Clones producing the Eimeria proteins of the invention can be identified using suitably labeled antibodies specific for the proteins. Monoclonal antibodies, which are preferred, can be prepared using standard methods as follows.

Antigenic proteins from Eimeria tenella are used to immunize animals such as mice, rats, horses, sheep, pigs, rabbits, etc., to obtain antibody producing somatic cells for fusion to myeloma cells.

Somatic cells with the potential for producing antibody, particularly B cells, are suitable for fusion with a myeloma cell line. These somatic cells may be derived from the lymph nodes, spleens and peripheral blood of primed animals. In the preferred embodiment of this invention mouse spleen cells are used, in part because these cells produce a relatively high percentage of stable fusions with mouse myeloma lines. It would be possible, however, to use rat, rabbit, frog or other cells instead.

Specialized myeloma cell lines have been developed from lymphocytic tumors for use in hyridoma-producing fusion procedures [Köhler and Milstein, Eur. J. Immunol. 6:511 (1976); Shulman et al., Nature 276:269 (1978); Volk et al., J. Virol. 42:220 (1982)]. These cell lines have been developed for at least three reasons. The first is to facilitate the selection of fused hybridomas among unfused and similarity indefinitely self-propagating myeloma cells. Usually, this is accomplished by using myelomas with enzyme deficiencies that render them incapable of growing in certain selective media that support the growth of hybridomas. The second reason arises from the inherent ability of lymphocytic tumor cells to produce their own antibodies. The purpose of using monoclonal techniques is to obtain fused hybrid cell lines with unlimited lifespans that produce the desired single antibody under the genetic control of the somatic cell component of the hybridoma. To eliminate the production of tumor cell antibodies by the hybridomas, myeloma cell lines incapable of producing light or heavy immunoglobulin chains or deficient in antibody secretion mechanisms are used. A third reason for selection of these cell lines is for their suitability and efficiency for fusion.

Many myeloma cell lines may be used for the production of fused cell hybrids, including, e.g., P3/X63-Ag 8, P3/NSI/1-Ag 4-1, SP2/O-Ag-14 and S194/5.XXO.BU.1. The P3/X63-Ag 8 and P3/NSI/1-Ag 4-1 cell lines have been described by Köhler and Milstein [Eur. J. Immunol. 6:511, (1976)]. Shulman et al. [Nature 276:269 (1978)] developed the Sp2/0-Ag14 myeloma line. The S194/5.XXO.BU.1 line was reported by Trowbridge [J. Exp. Med. 148:313 (1979)]. In the example of the present invention, the PAI-O mouse cell line (a non-Ig-producing subclone of P3/X63-Ag 8) was used.

Methods for generating hybrids of antibody-producing spleen or lymph node cells and myeloma cells usually involve mixing somatic cells with myeloma cells in a 10:1 proportion (although the proportion may vary from about 20:1 to about 1:1), respectively, in the presence of an agent or agents (chemical, viral or electrical) that promote the fusion of cell membranes. It is preferred that the same species of animal serve as the source of the somatic and myeloma cells used in the fusion procedure. Fusion methods have been described by Kohler and Milstein [Nature 256:495 (1975) and Eur. J. Immunol. 6:511 (1976)], by Gefter et al. [Somatic Cell Genet. 3:231 (1977)], and by Volk et al. (J. Virol. 42:220 (1982)]. The fusion-promoting agents used by those investigators were Sendai virus and polyethylene glycol (PEG). The fusion procedure for the example of the present invention uses PEG.

Because fusion procedures produce viable hybrids at very low frequency (e.g., when spleens are used as a source of somatic cells, only one hybrid is obtained for roughly every 1 x 10- spleen cells), it is essential to have a means of selecting the fused cell hybrids from the remaining unfused cells, particularly the unfused myeloma cells. A means of detecting the desired antibody-producing hybridomas among other resulting fused cell hybrids is also necessary.

Generally, the selection of fused cell hybrids is accomplished by culturing the cells in media that support the growth of hybridomas but prevent the growth of the myeloma cells, which normally would go on dividing indefinitely. (The somatic cells used in the fusion do not maintain long-term viability in in vitro culture and hence do not pose a problem). In the example of the present invention, myeloma cells lacking hypoxanthine phosphoribosyl transferase (HPRT-negative) were used. Selection against these cells is made in hypoxanthine/aminopterin/thymidine (HAT) medium, a medium in which the fused cell hybrids survive due to the HPRT-positive genotype of the spleen cells. The use of myeloma cells with different genetic deficiencies (drug sensitivities, etc.) that can be selected against in media supporting the growth of genotypically competent hybrids is also possible.

Several weeks are required to selectively culture the fused cell hybrids. Early in this time period, it is necessary to identify those hybrids which produce the desired antibody so that they may subsequently be cloned and propagated. Generally, around 10% of hybrids obtained produce the desired antibody, although a range of from 1 to 30% is not uncommon. The detection of antibody-producing hybrids can be achieved by any one of several standard assay methods, including enzyme-linked immunoassay and radioimmunoassay techniques which have been described in the literature [see, e.g., Kennet et al. (editors), Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses, pp. 376-384, Plenum Press, New York (1980)]. Several detection methods were used in the example of the present invention.

Once the desired fused cell hybrids have been selected and cloned into individual antibody-producing cell lines, each cell line may be propagated in either of two standard ways. A suspension of the hybridoma cells can be injected into a histocompatible animal. The injected animal will then develop tumors that secrete the specific monoclonal antibody produced by the fused cell hybrid. The body fluids of the animal, such as serum or ascites fluid, can be tapped to provide monoclonal antibodies in high concentration. Alternatively, the individual cell lines may be propagated in vitro in laboratory culture vessels. The culture medium containing high concentrations of a single specific monoclonal antibody can be harvested by decantation, filtration or centrifugation.

As produced in E. coli, the Eimeria proteins remain in the cytoplasm, or in inclusion bodies. To free the proteins it is thus necessary to disrupt the outer membrane. This is preferably accomplished by sonication, or by other mechanically disruptive means, such as a French pressure cell or Gaulin homogenizer.

Cell disruption could also be accomplished by chemical or enzymatic means. Since divalent cations are often required for cell membrane integrity, treatment with appropriate chelating agents such as EDTA or EGTA might prove sufficiently disruptive to facilitate the leakage of the proteins from the cells. Similarly, enzymes such as lysozyme have been used to achieve the same result. That enzyme hydrolyzes the peptidoglycan backbone of the cell wall.

The application of osmotic shock could also be employed. Briefly, this could be accomplished by first placing the cells in a hypertonic solution which would cause them to lose water and shrink. Subsequent placement in a hypotonic "shock" solution would then lead to a rapid influx of water into the cells with an expulsion of the desired proteins.

Once freed from the cells, the Eimeria proteins may be concentrated by precipitation with salts such as sodium or ammonium sulfate, ultrafiltration or other methods well known to those skilled in the art. Further purification could be accomplished by conventional protein purification techniques including but not limited to gel filtration, ion-exchange chromatography, preparative disc-gel or curtain electrophoresis, isoelectric focusing, low temperature organic solvent fractionation, or countercurrent distribution. Purification is preferably carried out, however, by immunoaffinity chromatography as described below.

The proteins of this invention or fragments thereof can also be chemically synthesized by a suitable

method such as by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. Solid phase synthesis as described by Merrifield [J. Am. Chem. Soc. 85:2149 (1963)] is preferred.

Such synthesis is carried out with amino acids that are protected at the alpha-amino-terminus. Trifunctional amino acids with labile side-chains are also protected with suitable groups which will prevent a chemical reaction from occurring at that site during the assemblage of the peptide. The alpha-amino protecting group is selectively removed to allow subsequent reaction to take place at the amino-terminus. The conditions for the removal of the alpha-amino protecting group do not cause deprotection of the side-chain protecting groups.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups (e.g., formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (e.g., benzyloxycarbonyl (Cbz) and substituted benzyloxycarbonyl), aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (e.g., benzyl, triphenylmethyl). The preferred protecting group is Boc. The side-chain protecting groups for Tyr include tetrahydropyranyl, tert.-butyl, triyl, benzyl, Cbz, 4-Br-Cbz and 2,6-dichlorobenzyl. The preferred side-chain protecting group for Tyr is 2,6-dichlorobenzyl. The side-chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl and cyclohexyl. The preferred side-chain protecting group for Asp is cyclohexyl. The side-chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl and Cbz. The preferred protecting group for Thr and Ser is benzyl. The side-chain protecting groups for Arg include nitro, Tos, Cbz, adamantyloxycarbonyl or Boc. The preferred protecting group for Arg is Tos. The side-chain amino group of Lys may be protected with Cbz, 2-ClCbz, Tos or Boc. The 2-Cl-Cbz group is the preferred protecting group for Lys. The selection of the side-chain protecting group is based on the following: The side-chain protecting group remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling conditions. The side-chain protecting group must be removable upon the completion of the synthesis of the final peptide, using reaction conditions that will not alter the target peptide.

Solid phase synthesis is usually carried out from the carboxy-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is formed when the attachment is made to a chloromethylated or hydroxymethyl resin and the resultant target peptide will have a free carboxyl group at the C-terminus. Alternatively, a benzhydrylamine or p-methylbenzhydrylamine resin is used in which case an amide bond is formed and the resultant target peptide will have a carboxamide group at the C-terminus. These resins are commercially available and their preparation is described by Stewart et al., "Solid Phase Peptide Synthesis" (2nd Edition, Pierce Chemical Co., Rockford, IL., 1984).

The C-terminal amino acid, Arg, protected at the side-chain with Tos and at the alpha-amino function with Boc is coupled to the benzhydrylamine resin using various activating agents including dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide and carbonyldiimidazole. Following the attachment to the resin support the alpha-amino protecting group is removed by using trifluoroacetic acid (TFA) or HCl in dioxane at a temperature between 0° and 25° C. Dimethylsulfide is added to the TFA after the introduction of methionine (Met) to suppress possible S-alkylation. After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the required order to obtain the desired peptide sequence.

Various activating agents can be used for the coupling reactions including DDC, N,N'-diisopropylcarbodiimide, benzotriazol-1-yl-oxy-tris-(dimethylamino)-phosphonium hexafluorophosphate (BOP) and DCC-hydroxybenzotriazole (HOBt). Each protected amino acid is used in excess (>2.5 equivalents), and the couplings are usually carried out in DMF, CH_2Cl_2 or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage by the ninhydrin reaction as described by Kaiser et al., Anal. Biochem. 34:595 (1970). In cases where incomplete coupling is determined the coupling reaction is repeated. The coupling reactions can be performed automatically on a Vega 250, Applied Biosystems synthesizer or other commercially available instrument. After the entire assemblage of the target peptide, the peptide-resin is deprotected with TFA/dithioethane and then cleaved with a reagen such as liquid HF for 1-2 hours at 0° C which cleaves the peptide from the resin and removes all side-chain protecting groups.

Side-chain to side-chain cyclization on the solid support requires the use of an orthogonal protection scheme which enables selective cleavage of the side-chain functions of the acidic amino acids (e.g., Asp) and the basic amino acids (e.g., Lys). The 9-fluorenylmethyl (OFm) protecting group for the side-chain of Asp and the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group for the side-chain of Lys can be used for this purpose. In these cases the side-chain protecting groups of the Boc-protected peptide-resin are selectively removed with piperidine in DMF. Cyclization is achieved on the solid support using various

activating agents including DCC, DCC/HOBt or BOP. The HF reaction is carried out on the cyclized peptide-resin as described above.

Purification of the synthetic proteins can be carried out as described above for the recombinantly produced proteins.

Eimeria proteins can also be recovered from extracts of membrane proteins from E. tenella or other Eimeria species by immunoprecipitation or immunoaffinity chromatography. As already noted, such methods can produce the complete, wild-type proteins. In some cases, these proteins are larger than the proteins produced by recombinant DNA methodology. Monoclonal antibodies for this purpose can be produced as described above, using synthetic or natural Eimeria proteins as the antigen.

Other useful proteins which have the necessary immunoreactive and/or antigenic determinants are antibodies or fragments thereof which are anti-idiotypic toward the active determinant or determinants on the proteins of the invention. Such anti-idiotypic antibodies can be raised against other antibodies which are specific for the determinants on the proteins of the invention (i.e., the anti-idiotypic antibodies are anti-antibodies). Preferably, monoclonal anti-idiotypic antibodies are used. Such antibodies can be administered as a vaccine, in the same manner that the Eimeria proteins themselves can be used.

One or more of the Eimeria proteins and anti-idiotype antibodies of this invention can be formulated into vaccines comprising the proteins and a physiologically acceptable carrier. Suitable carriers include, e.g., 0.01 to 0.1 M phosphate buffer of neutral pH or physiological saline solution.

Enhanced immunity against coccidiosis can be produced in one of two ways. First, an adjuvant or immunopotentiator can be added to the vaccine. Secondly, the proteins of the invention can be presented to an animal that is to be immunized in a larger form, either as a cross-linked complex or conjugated to a carrier molecule.

Suitable adjuvants for the vaccination of animals include but are not limited to Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyldioctadecylammonium bromide, N,N-dioctadecyl-N′,N′-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The proteins could also be administered following incorporation into liposomes or other microcarriers.

Incorporation into liposomes or other microcarriers provides a means by which the release of the vaccines can be sustained over a prolonged period of time. A pump such as an Alza pump could be used for the same purpose.

The immunogenicity of the proteins of the invention, especially the smaller fragments, can be enhanced by cross-linking or by coupling to an immunogenic carrier molecule (i.e., a macromolecule having the property of independently eliciting an immunological response in a host animal, to which the proteins and protein fragments of the invention can be covalently linked). Cross-linking or conjugation to a carrier molecule may be required because small protein fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, i.e., they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders the fragments immunogenic through what is commonly known as the "carrier effect".

Suitable carrier molecules include, e.g., proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides etc. A useful carrier is a glycoside called Quil A, which has been described by Morein et al., Nature 308:457 (1984). Protein carrier molecules are especially preferred, including but not limited to mammalian serum proteins such as keyhole limpet hemocyanin, human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in the art. Preferably, but not necessarily, the protein carrier will be foreign to the host animal in which antibodies against the Eimeria proteins are to be elicited.

Covalent coupling to the carrier molecule can be carried out using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the proteins or fragments of the invention can be coupled, e.g., using water soluble carbodiimides such as dicyclohexylcarbodiimide, or glutaraldehyde.

Coupling agents such as these can also be used to cross-link the proteins and fragments to themselves without the use of a separate carrier molecule. Such cross-linking into protein or protein fragment aggregates can also increase immunogenicity.

Administration of an effective amount of the vaccines of this invention can protect poultry against infection by E. tenella. Monoclonal antibodies against the E. tenella antigens cross-react with E. acervulina and E. maxima in vitro, indicating that protection may also be conferred against these species. An effective

dose of the proteins or protein fragments ranges from about 10 to about 50 micrograms/kg of body weight of the vaccinated animal. A dose of about 25-50 µg/kg is preferred. Initial vaccinations are preferably followed by booster vaccinations given from one to several weeks later. Multiple boosters may be administered. The dosages of such boosters generally range from about 5 to 50 µg/kg, preferably about 20-50 µg/kg. Standard routes of administration can be used such as subcutaneous, intradermal, intramuscular, oral, anal or in ovo administration.

The presentation of the coccidial antigens of the invention to the immune systems of fowl can be achieved by cloning genes coding for the antigens into bacteria (e.g., E. coli or Salmonella) or into viruses (e.g., poxviruses or herpesviruses) and administering the live vector systems to the birds orally, by injection or by other commonly used routes. Carbit et al. [in: Vaccines, 1987, Cold Spring Harbor Laboratory, pp. 68-71] have described the use of E. Coli, while Clements [Pathol. Immunopathol. Res. 6:137 (1987)] has described the use of Salmonella. Moss et al. [Ann. Rev. Immunol. 5:305 (1987)] have reviewed the use of viral vector systems employing recombinant poxviruses.

One kind of poxvirus, vaccinia virus, can be used to test the delivery of coccidial antigens in cell culture and in animals. For analytical studies, vaccinia virus has been found to be more efficient than fowlpox virus, another poxvirus carrier that can be used. This is because vaccinia virus multiplies more rapidly than the avian virus and has a host range that is not restricted to chicken cells. Large amounts of heterologous DNA can be inserted into the vaccinia viral genome without inhibiting viral maturation and infectivity [Smith et al., Gene 25:21 (1983)]. The insertion and expression of multiple heterologous genes using the virus elicits antibody production against expressed antigens in infected animals [Perkus et al., Science 229:981 (1985)].

The techniques used to produce recombinant vaccinia viruses can be readily adapted by routine procedures to fowlpox or herpesvirus systems. The use of such recombinant viruses as carriers in vaccines against coccidiosis is especially advantageous in that vaccinated fowl develop immunity against both the coccidial antigen and the viral carrier (i.e., such vaccines are bivalent). The utility of such vaccines can be further enhanced by inserting additional genes into the carrier virus. For example, parts of the Newcastle disease viral genome can be inserted together with a coccidial antigen gene into a fowlpox virus, thereby conferring immunity against Newcastle disease, coccidiosis and fowlpox, all with a single vaccine.

The administration of the live vector vaccines of the invention can be carried out by numerous methods well known in the art. For example, the "stick" method commonly used to vaccinate poultry against fowlpox virus can be used. This method consists of sticking or pricking the skin of the wing web with a sharp needle dipped into the vaccine. The needle usually has an eye near the tip like a sewing machine needle which carries a drop of vaccine. Alternatively, the live vaccines can be injected subcutaneously or intradermally into the wing web or any other site.

The recombinant live vector vaccines can also be added to drinking water or even sprayed over chicks that are to be vaccinated. They can also be administered in feed, preferably after protective encapsulation [Balancou et al., Nature 322:373 (1986)], or in ovo. In the latter method, the viral vaccines are injected directly into chicken embryos [Sharma, Avian Dis. 25:1155 (1985)].

EXAMPLE

Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively.

1. PREPARATION OF MONOCLONAL ANTIBODIES AGAINST EIMERIA ANTIGENS

1.1. PARASITE PREPARATION

Sporozoites of E. tenella, E. acervulina, E. brunetti, and E. maxima were isolated from sporulated oocysts by standard procedures. Briefly, sporulated oocysts were washed with distilled water and 20% bleach and then with distilled water. The oocysts were disrupted in a tissue homogenizer and insoluble material, including sporocysts, was recovered by centrifugation. The released sporocysts and other material in the pellet were resuspended in 0.25% trypsin and chicken bile in Hank's salt solution, pH 8, and incubated for 2 hours at 40°C. The excising solution was removed by two washes with RPMI-1640 medium

containing 10% fetal bovine serum (FBS), followed by two washes with PBS at pH 7.4.

The sporozoites were then purified over a metrazimide gradient [Wisher et al., Parasitiology 88:515 (1984)]. Briefly, the sporozoites were resuspended in 2 ml of PBS, pH 7.0, and 1 ml of the suspension was layered over a 15 ml metrizamide gradient. The gradient was composed of 5 ml of each of 12%, 18% and 24% metrazimide in PBS, pH 7.0. The sporozoites were sedimented by centrifugation at 900 X g for 40 minutes. Purified sporozoites were isolated from the interface between the 18% and 24% metrizamide by insertion of a 21 gauge needle through the side of the tube and aspirating the sporozoites into a syringe.

The purified sporozoites were washed 3 times with PBS, pH 7.0 and used immediately for immunizations, infection studies, surface labeling with ^{125}I, immunofluorescence assays or SDS-polyacrylamide gel electrophoresis [Laemmli, Nature 227:680 (1970)] and Western blotting studies.

Merozoites of E. tenella were isolated as described below in Section 6.2.3. The purified merozoites were used for immunizations and were solubilized with Laemmli sample buffer for SDS-polyacrylamide gel electrophoresis and Western blotting studies.

1.2. IMMUNIZATIONS

Eight female Balb/c mice (Charles River, Wilmington, Mass.) were immunized with purified live sporozoites according to the following schedule.

Day 1	1×10^7 sporozoites intravenously (i.v.)
Day 7	6×10^6 sporozoites intraperitoneally (i.p.)
Day 85	6×10^6 sporozoites i.p.
Day 120	3×10^7 sporozoites i.p.
Day 244	Pre-fusion immunization boosters
Day 1	5×10^6 sporozoites i.v., 5×10^6 sporozoites i.p.
Day 2	same as Day 1
Day 5	fusion of hyperimmune splenocytes and myeloma cells

The serum from each mouse was tested for anti-sporozoite antibodies by ELISA with purified sporozoite proteins, by Western blotting assays with solubilized sporozoite proteins, by immunoprecipitation of ^{125}I-labeled sporozoite surface proteins, and by immunofluorescence assays with purified sporozoites. The mouse with the highest sporozoite antibody reactivity (mouse 107-2) was chosen for the pre-fusion immunization boosters (see Fig. 1 for ELISA analysis of this antiserum). On the fifth day, the mouse was killed and the spleen was removed for the preparation of splenocytes.

1.3. CELL CULTURE AND CELL FUSIONS

Two days before fusion, splenocyte feeder cells were prepared from naive mice in complete medium [Iscove's modified Dulbecco's medium (IMDM, Gibco) with 10% FBS, glutamine (2.0 mM), and 2-mercaptoethanol (100 µM)] plus HAT (100 µM hypoxanthine, 0.4 µM aminopterin and 16 µM thymidine). Using a modification of the procedure of de St. Groth et al. [J. Immunol. Methods 35:1 (1980)], 10^8 spleen cells were fused with 10^8 PAI-O mouse myeloma cells. Any other myeloma cell suitable for the preparation of hybridomas could be used. A number of such myeloma cells are known and available to the man skilled in the art.

The cells were mixed, pelleted by centrifugation and resuspended under constant gentle agitation in 1.0 ml of 35% (vol/vol) polyethylene glycol in IMDM at 37° C over 1 minute. After 3 minutes of incubation at 37° C, the cells were pelleted again and gently resuspended in 10 ml of IMDM + HAT. The cells were then diluted to 1×10^6 cells/ml in complete medium + HAT and dispersed into 24-well microtiter plates (1 ml/well) containing 5×10^5 splenocyte feeder cells in 1 ml of complete medium.

Hybridoma supernatants were assayed for anti-sporozoite antibodies by ELISA with purified sporozoites, by Western blotting with sporozoite proteins, by immunoprecipitation with ^{125}I-labeled sporozoite surface proteins and by immunofluorescence with purified sporozoites and with sporozoite-infected cells. The hybridomas were cloned by limiting dilution.

1.4. SPOROZOITE ELISA

Purified sporozoites (4×10^4) were added to each well of a 96-well U-bottom PVC plate which had previously been blocked with 1% BSA in PBS, pH 7.0. The sporozoites were sedimented to the bottom of the wells by centrifugation at 1000 x g for 5 minutes. The sporozoites were resuspended in 100 µl of diluted antiserum or hybridoma supernatants and incubated for 2 hours at room temperature with constant agitation. The sporozoites were then washed with 1% BSA in PBS, pH 7.0, to remove unbound antibody.

To detect specific antibody bound to the sporozoites, 100 µl of peroxidase-conjugated goat anti-mouse IgG were added to the resuspended sporozoites, and the suspension was incubated for 2 hours at room temperature. The sporozoites were washed, and bound antibody was visualized by adding substrate solution (o-phenylenediamine, 0.4 mg/ml in 0.1 M citrate buffer, pH 4.5., 0.12% hydrogen peroxide) for 30 minutes at room temperature. The reaction was stopped by the addition of 2.5 M H_2SO_4 containing 50 mM sodium metabisulfite. The amount of bound antibody was determined by reading the OD_{488} of the substrate color.

From a total of 480 wells plated from the cell fusion, 432 were positive for hybridoma growth. Of these, 358 hybridomas tested positive for antibody production in the primary sporozoite ELISA. During expansion and passage of these original parental hybridoma cells, 104 died or stopped producing antibody and thus were negative in subsequent screenings with the sporozoite ELISA and Western blot assays. The sporozoite ELISA identified 205 hybridomas which were producing antibody at 10X background levels.

1.5. WESTERN BLOTTING OF SPOROZOITE PROTEINS

Purified sporozoites (approximately 5×10^7 sporozoites per ml per gel) were solubilized in Laemmli sample buffer, separated by SDS-polyacrylamide gel electrophoresis in either a 12.5% gel or a 7.5 to 20% gradient gel (Laemmli, supra) and electrophoretically transferred to nitrocellulose sheets. The sheets were blocked in 3% gelatin buffer (3% gelatin, Tris-HCl, pH 7.5, 0.15 M NaCl) and cut into strips, and the strips were allowed to react with diluted antiserum or hybridoma supernatant for 12 hours at 4° C in 1% BSA buffer (1% BSA, 50 mM sodium phosphate, pH 6.5, 0.5 M NaCl, 0.05% Tween-20). The strips were washed in PBS, pH 7.4, 0.05% Tween-20 and the specifically bound antibody was detected with a peroxidase-conjugated anti-mouse antibody. The bound antibodies were visualized by adding substrate solution [4-chlorp-1-naphthol (30 mg dissolved in 10 ml of ice cold methanol and 50 ml of Tris-HCl, pH 7.5), 0.15 M NaCl, 0.015% final concentration H_2O_2] for 30 minutes at room temperature. The reaction was terminated by extensive washing with distilled water.

Of the antibodies that were positive in the sporozoite ELISA, 160 were also positive by Western blotting analysis using solubilized sporozoite proteins.

Western blot analysis (see Fig. 2) showed that the monoclonal antibodies fell into one of three reactivity patterns: (a) those which bind single Eimeria proteins (e.g., 11A1 and 11D1), (b) those which bind to 2 or 3 proteins (e.g., 6A5 and 20C6) and (c) those which bind to multiple proteins (e.g., 11A5, 13A6 and 14B5).

The antibodies were further characterized by Western blot analysis using E. tenella merozoite and E. acervulina sporozoite proteins (Fig. 3). A number of antibodies, including 3A5, 13A6, 7D1 and 20C6, recognized proteins isolated from sporozoites of E. tenella and E. acervulina and from merozoites of E. tenella. Other antibodies, such as 6A5, were shown to be species and stage specific and to bind only to proteins from E. tenella sporozoites.

A summary of results obtained on some of the antibodies is shown in Table 1, in which the specificity of the antibodies is shown both in terms of (a) the origin and size of the protein(s) in the gels to which the antibodies bound and (b) the size of ^{125}I-labeled Eimeria tenella proteins precipitated by the antibodies (right column). The antibodies are further characterized in the Table by isotype.

TABLE 1

WESTERN BLOT ANALYSIS						
Antibody	Isotype	Eimeria Protein (gel size in kd)				Size of Protein Ppt.
		Tenella		Acervulina	Maxima	
		Spz	Mrz	Spz	Spz	(kd)
7B2	G_2a	>200	-	-	-	-
7D4	G_1	120	120	120	-	110
7D1	G_1	120	120	120	N.D.	110
20C6	G_1	120	120	120	N.D.	110
3A5	M	120	120	120	17	120
19D6	G_3	180	180	-	-	120
8A2	G_{2a}	37	37	-	-	37
6A5	G_{2b}	28/26	-	-	-	25
14B5	N.D.	>150	N.D.			N.D.
15B3	N.D.	>150	N.D.			N.D.
14B1	G_3	6	6	-	-	24/17
12B2	G_3	28/26	-	-	-	24/17
15A3	G_1	28/6	-	-	-	17/15/6
15C4	M	28/26	-	-	-	105/15/6
12C3	G_3	28	-	N.D.	N.D.	25
5B6	G_3	-	N.D.			6
3C4	M	m	m	m	-	70
16D2	M	m	m	m	-	70/85
13A6	M	m	m	m	-	110
11B6	G_3	m	m	m	-	105
12A3	G_3	m	m	m	-	24/17
12D4	G_1	m	N.D.			N.D.

Spz and Mrz are abbreviations for sporozoite and merozoite, respectively.

G and M refer to IgG and IgM, respectively.

m indicates that the antibodies bound to multiple proteins ranging from 24 to more than 200 kd in size.

Values indicated by N.D. were not determined.

1.6. IMMUNOPRECIPITATION OF ^{125}I-LABELED SPOROZOITE SURFACE PROTEINS

The surface proteins of purified sporozoites were labeled with ^{125}I by the IODOGEN method (Pierce Chemical Co.) or by use of IODOBEADS (Pierce Chemical Co.). For the latter procedure, 4 IODOBEADS were washed 3 x with 0.2 M sodium phosphate, pH 7.5, and 1-3 mCi of ^{125}I-Na were added and incubated for 5 minutes at room temperature. Purified sporozoites (3×10^8) in 200µl of PBS, pH 7.0, were added to the reaction vial, and the incubation was continued for 15 minutes. At the end of the incubation, phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 0.5 mM.

The sporozoites were recovered from the incubation mixture by centrifugation at 12,000 x g for 30 seconds and solubilized in 1 ml of either 2% sodium dodecysulfate (SDS) or 1% Triton X-100 in PBS, pH 7.0. Insoluble material was removed by centrifugation for 3 minutes at 12,000 x g. The solubilized sporozoite proteins were dialyzed against 3 liters of PBS, pH 7.0, at 4° C using a 3,500 molecular weight cutoff membrane to remove any residual free ^{125}-I. The ^{125}I-labeled sporozoite proteins (typically 1.5×10^8 cpm incorporated into protein) were stored at 4° C until used. The TCA precipitable radioactivity was typically in excess of 95% of the total radioactivity. SDS polyacrylamide gel electrophoretic analysis of the ^{125}I-labeled sporozoite proteins is shown in Fig. 4, left panel.

Immunoprecipitation was carried out by adding 300 µl of hybridoma supernatant or diluted antiserum to

250 µl of ^{125}I-labeled sporozoite proteins (1 x 10^5 cpm) in Buffer I (0.25% NP-40, 10 mM Tris-HCl, pH 7.5, 0.15 M NaCl). Following incubation for 16 hours at 4° C, 100-200 µl of a 50% suspension of goat anti-mouse IgG coupled to agarose (Sigma Chemical Co.) were added, and the mixture was incubated on a rotating mixer for 2 hours at room temperature. The beads were pelleted by centrifugation for 1 minute at 12,000 x g and washed 3x in Wash Buffer (0.1% SDS, 0.5% NP-40, 0.2% sodium deoxycholate, 10 mM PMSF, 10 mM Tris-HCl, pH 8.5, 0.15 M NaCl).

The ^{125}I-labeled proteins bound to the solid phase antibodies were released and denatured by adding 60 µl of 2 x Laemmli sample buffer and heating for 3 minutes at 95° C. The immunoprecipitated ^{125}I-labeled sporozoite proteins were separated by SDS-polyacrylamide gel electrophoresis in a 12.5% gel and visualized by autoradiography.

The results of the immunoprecipitation assay with the immune mouse serum are shown in Fig. 4, right panel. Of the hybridoma antibodies that were positive by sporozoite ELISA, 74 were positive by immunoprecipitation assay. As shown in Fig. 5, the hybridoma antibodies fell into two categories, those which precipitated only single proteins (e.g., 3C4, 6A5, 7D4, 8A2, 11D2 and 20C6), and those which precipitated two or more proteins (e.g., 12B2, 15A3, 15C4 and 19D6).

1.7. IMMUNOFLUORESCENCE ASSAYS WITH PURIFIED SPOROZOITES

Sporozoites (1 x 10^5) were added to 8-chambered slides (Lab Tek) in PBS, pH 7.0, and air dried at 37° C for 12 hours. The slides were blocked with 10% normal goat serum for 2 hours at 37° C. Diluted antiserum or hybridoma supernatant were added to each chamber and incubated for 2 hours at room temperature. The slides were washed, and a rhodamine-conjugated anti-mouse antibody (diluted in PBS, pH 7.0, 0.3% Triton X-100) was added for 1 hour at room temperature. After washing the slides, the bound antibody was visualized by fluorescence.

Most of the antibodies showed specific immunofluorescence either to the surface membrane and/or to the refractile body of air-dried sporozoites (Fig. 6, panels A and B). Some antibodies intensely stained the apical tip of the sporozoite and only lightly stained the remaining sporozoite surface (Fig. 6, panel C). A representation of the air-dried purified sporozoites can be seen in Fig. 6, left hand slides of panels A, B, C and D. The purified sporozoites were intact and elongated and showed the prominant large posterior refractile body (PRB) and the smaller anterior refractile body (ARB). The apical end (A) of the sporozoite was opposite the posterior refractile body. There was also slight contamination of the preparations by intact sporocysts (panel B, left slide) and broken sporocyst membranes.

1.8. SUMMARY OF ELISA, WESTERN BLOT, IMMUNOPRECIPITATION AND IMMUNOFLUORESCENCE RESULTS

A summary of results from the above analyses of 55 monoclonal antibodies is shown in Table 2.

TABLE 2

SUMMARY OF MONOCLONAL ANTIBODY ANALYSES

Antibody	WESTERN BLOT: E. tenella Spz.[a]	Low Spz.[b]	E. acervulina Spz.[c]	E. tenella Mz.[d]	IFA[e]	Immuno-ppt.[f]
3C4	M^1				-	60-80
11B6	M^1	+	+	+	1,2,4	105
12A5	M^1	-	-	-	1	-
14D4	M^1	+	+	+	1,3,4	66
15B6	M^1				1,4,7	20-24
17A5	M^1		+	+	1	150/83
18B6	M^1	+	+	+	-	25/20, 66/60
19C6	M^1	+	+	+	1,2	25/20
20A2	M^1	+	+	+	5	66/60
20B4	M^1	+	+	+	1,4	86/60
11C4	M^2		+		1,6	-
12A3	M^2	-	-	-	1,4,7	22/24
13A6	M^2	+	+	+	1,5	110
14B6	M^2				1,4,7	105-120
14D1	M^2				-	120
9B2	M^3	+	+	+	5	66/45
12B1	M^3	+	-	-	6	26-28
14C6	M^3	-	-	-		105
15C4	M^3	+	-	-	6	105
16D2	M^3				-	60-80
20C3	M^3	+	+	+	3	14-17
3A5	120	+	+	+	3	-
6A4	120				-	-
7D1	120		+	+	1,2,4	110
7D4	120		+	+	5,1	110
10A6	120	+	-		1,2,6	105
11D2	120	-	-	-	4,1,2	105
14A1	120	-	-	-	6,1	110
17B6	120	-	-		1,6,7	120
17C6	120		-	-	8,1	105
19D6	120	+	-	-	3	120
20C6	120	+	+	+	1,2	110

TABLE 2 (continued)

	WESTERN BLOT					
Antibody	E. tenella Spz.[a]	Low Spz.[b]	E. acervulina Spz.[c]	E. tenella Mz.[d]	IFA[e]	Immuno-ppt.[f]
10A5	>150		-		7,5	105
11A6	>150	-	-	-	3	-
7B2	>200	+			-	>200
11B1	>150,200	-	-	-	1,7,6	27
11D4	120/24	+	-	-	1	27
11D6	120/24	+	-	-	2	-
12C3	120/24	+	-	-	1,8,2	25
15B2	120/24	+	+		3	-
15A3	90/10-14	+	-	-	1,6	28/14-17
14C3	60	-	-	-	1,4	6
14A5	120/6	-	-		1,3,6	6
8A2	37	+	+	-	1,4	37
6A5	28, 10-14	+	-		1,6	25-28
11A1	24	+	-	-	1,6	-
11C1	24	+	-	-	-	-
12B2	24	+	-	-	1,5	24/120
12C6	24	+	-	-	-	-
16B1	24	+	-	-	1,4	6/14-17
18D5	24	+	-	-	1,6	48/25/6
20C4	24				1,3	5/14-17
14B1	< 6	+	-	-	1,6	20-24
10A2	-	-			1,2,4	6/105
5B6	-				1,6	6/17/15

a. Values shown are the molecular weights of E. tenella sporozoite (Spz.) proteins recognized by the antibodies in Western blots or groups of recognized proteins having molecular weights of 40-150 kd (M^1), 120 and 80-150 kd (M^2) and 25 and 40-150 kd (M^3).

b. Western blot assays, were performed with 1/5 the usual amount of E. tenella sporozoite (Spz.) protein. Antibodies showing a positive reaction are thus of higher affinity.

c. Western blot reactivity is shown against E. acervulina sporozoite (Spz.) proteins.

d. Western blot reactivity is shown against E. tenella merozoite (Mz.) proteins.

e. Immunofluorescence assay (IFA) staining pattern results are summarized for indirect assay of air-dried E. tenella sporozoites as (1) surface, (2) tip, (3) patchy surface, (4) bright surface, (5) light surface, (6) diffuse surface, (7) refractile body and (8) punctate staining.

f. Molecular weights of ^{125}I-labeled E. tenella sporozoite proteins captured by the antibodies in immunoprecipitation (Immunoppt.) assays are shown.

Monoclonal antibodies 7D4, 7D1, 20C6, 8A2, 6A5 and 7B2, which are preferred, have been deposited in the form of hybridoma cells secreting these monoclonal antibodies with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, U.S.A. under the provisions of the Budapest Treaty and assigned accession Nos. HB 9707, HB 9708, HB 9709, HB 9710, HB 9711 and HB 9712, respectively.

1.9. IN VITRO INFECTION ASSAYS

Primary chicken kidney epithelial cells were established according to the method of Doran et al., J. Protozool. 25:544 (1978) and grown to 40-50% confluency in 4-chambered Lab-Tek slides. MDBK (Madin-Darby bovine kidney) cells (ATTC-CCL 22) were also used in place of the chicken kidney epithelial cells.

The cells were inoculated with 50,000 or 200,000 purified sporozoites. At 16 hours post-infection, the cell monolayers were washed several times to remove any sporozoites which had not penetrated the cells. Representative inoculated cell cultures were fixed in 100% methanol (room temperature for 5 minutes) at 3, 16, 24, 48, 64, 96 and 120 hours post-infection Fixed slides were stored in 1% BSA in PBS, pH 7.0, at 4° C until processed for immunofluorescence as described above. Staining patterns obtained with various antibodies are shown in Fig. 7.

Between 3 and 24 hours after infection, the fixed cultures revealed intracellular sporozoites (Fig. 7, 7D4 at 3 hours and 8A2 at 19 hours). At later times, the sporozoites degenerated to refractile bodies only (7D4, 60 hrs). The surface and apical tip of the intracellular sporozoites stained brightly with antibody 7D4 (Fig. 7, 7D4 at 3 hours), but this antibody did not stain the surface of the infected cells.

After 24 hours, the sporozoites began to degenerate and develop into schizonts that matured during the following 48 hours. Antibody 7D4 continued to react with the degenerating sporozoites but did not react with the immature schizonts (Fig. 7, 7D4, 60 hrs). As the schizonts matured, however, 7D4 began to react with structures within the schizonts (Fig. 7, 7D4, 100 hrs). These structures were the developing merozoites, and antibody 7D4 continued to react with a surface antigen of the mature and released merozoites (Fig. 7, 7D4, 120 hrs).

Thus, 7D4 identified a 120 kd membrane antigen which was present on E. tenella sporozoites and merozoites. This antigen was not expressed during the schizont stage of parasite development until immature merozoites developed within the schizonts.

Antibody 14B1 showed a pattern of reactivity similar to that of antibody 7D4, staining the surface and tip of the intracellular sporozoites (Fig. 8, 14B1, 16 hrs) and showing diffuse staining of the cytoplasm in the immediate vicinity of the intracellular sporozoite. The antigen recognized by 14B1 is present on the apical tip of the immature merozoite within the mature schizont (Fig. 8, 14B1, 100 hrs) and the apical tip of the mature released merozoites (Fig. 8, 14B1, 120 hrs). The staining patterns exhibited by antibodies 7D4 and 14B1 are similar, but the proteins these antibodies recognize have very different molecular weights of about 120 and 6 kd, respectively.

Although antibodies 7D4 and 14B1 reacted with most stages of parasite development, other antibodies reacted only with surface antigens (Fig. 7, 15A3) or with the refractile body (Fig. 7, 7A2) of intracellular sporozoites and not with the schizont or merozoite stages of the parasite.

Two unique antibodies, 8A2 and 19D6, were identified by the infection assay. Antibody 8A2 reacted with a 37 kd protein present on the surface of sporozoites (Fig. 7C, 8A2, 19 hrs), in all stages of the developing schizont (Fig. 7C, 8A2, 120 hrs) and on the surface of released merozoites (Fig. 7C, 8A2, 120 hrs). Unlike the proteins recognized by antibodies 7D4 and 14B1, the 37 kd protein was synthesized throughout the intracellular development of the parasite.

Antibody 19D6 reacted not only with a 180 kd sporozoite surface protein, but also with a protein in the cytoplasm of the sporozoite-infected cells (Fig. 8, 19D6 at 3 hours). The cytoplasmic protein recognized by antibody 19D6 might have been shed by the sporozoite after cell infection, since the protein disappeared during immature schizont development and reappeared in the mature schizont and in the released merozoites (Fig. 8B, 19D6, 120 hrs).

Serum antibodies from chickens which have survived an E. tenella infection stain the apical tip and surface of intracellular sporozoites (Fig. 8B, Immune Chick Sera, 3 hrs) in a pattern similar to the staining pattern of antibody 7D4, but not the refractile bodies of the intracellular sporozoite.

The immunofluorescence studies with sporozoite-infected chicken kidney cells identified antigens which were (a) specific to the sporozoite (e.g., the greater than 200 and 28 kd proteins recognized by antibodies 7B2 and 6A5, respectively), (b) found in all stages of the intracellular parasite (e.g., the 37 kd protein recognized by 8A2) and (c) specific to sporozoites and merozoites but not to the schizont (e.g., the 120 and 6 kd proteins recognized by antibodies 7D4 and 14B1, respectively).

1.10. IN VITRO SPOROZOITE NEUTRALIZATION ASSAYS

In a modification of the method of Schmatz et al., J. Protozool. 33:109 (1986), MDBK cells were trypsinized and suspended in Minimal Essential Medium (Gibco) supplemented with 1% FBS at a density of 7.5×10^4 cells/ml. To each well of a microtiter plate (tissue culture treated 96-well), 1.5×10^4 cells were added and incubated for 48 hours at 40° C. Purified sporozoites were either pretreated with antibody for 1 hour at 40° C or left untreated prior to infecting the cell monolayers. The antibodies (either tissue culture supernatants, ascites fluid or antiserum) were extensively dialyzed against PBS, pH 7.0, heat inactivated at 56° C for 30 minutes and sterile filtered before use.

Immediately after infection, [5,6]-^{3}H-uracil was added to all wells to give a final level of 5 µCi/ml. At 19 hours post-infection, the medium was removed and the cultures were washed once with PBS. The cells are released with trypsin-EDTA for 15 minutes at 40° C and harvested onto glass fiber filters. The filters were dried, placed in scintillation fluid (READY-SOLV®, New England Nuclear) and counted for bound radioactivity. The ability of the antibodies to inhibit sporozoite penetration and/or development was determined by the radioactivity incorporated into cells infected with untreated sporozoites, compared to cells infected with antibody-treated sporozoites.

Sporozoites were also preincubated with control antibodies, buffer or lasalocid, a coccidiostatic drug. Lasalocid completely blocks sporozoite development within the MDBK cells and greatly reduces the incorporation of ^{3}H-uracil.

The results are shown in Fig. 9, where it can be seen that antibodies 7D4 (□), 8A2 (○) and 14B1 (●) significantly inhibited ^{3}H-uridine incorporation into the Infected MDBK cultures. Antibody 6A5 (▫) was less effective but showed some inhibition. Treatment with buffer (Δ) and control antibody (X) produced no inhibition, while lasalocid (*) produced essentially complete inhibition.

2. CONSTRUCTION OF cDNA EXPRESSION LIBRARIES

2.1. PREPARATION OF SPORULATING OOCYSTS

Ceca were removed from 3-week-old chicks (Hubbard Cross; Avian Services, Frenchtown, New Jersey, U.S.A.) 7 days after oral inoculation with 50,000 E. tenella sporulated oocysts per bird and ground in a Waring blender with distilled water for 1 minute. The volume was adjusted to 1 liter with distilled water, and Pepsin (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was added to 3 g/l. The pH was adjusted to 2.0 with concentrated HCl, and the mixture was incubated and stirred for 2 to 3 hours at 39° C, or until a single

oocyst suspension was observed. After digestion, the pH was adjusted to 8.0 with 10 N NaOH, and 3 liters of distilled water were added. The mixture was allowed to settle overnight. The supernatant was then removed and the sediment was washed with water until the supernatant was clear. The oocysts were sporulated by bubbling air through the suspension in distilled water at room temperature. Sporulation was stopped after 24 hours for RNA preparation.

2.2. ISOLATION OF SPORULATING OOCYST mRNA

Total RNA was prepared by a modification of the guanidinium/cesium chloride method described by Maniatis et al., supra, page 196. The oocysts were washed with PBS (0.15 M NaCl, 20 mM sodium phosphate, pH 7.9) and resuspended by gentle vortex mixing in 10 ml of a solution containing 5 M guanidinium isothiocyanante, 50 mM Tris-HCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Sarkosyl (sodium N-lauroyl sarcosine, Sigma Chemical Co.) and 0.1 M β-mercaptoethanol, pH 7.4, with 5 µl of Antifoam A (Union Carbide, Danbury, Connecticut, U.S.A.) or another antifoaming agent preferably added. The cell suspension was homogenized until good oocyst breakage was observed microscopically.

Insoluble cellular debris was removed by low speed centrifugation, and the homogenate was divided into 4 aliquots and layered onto 1.2 ml of 5.7M CsCl, 0.1M EDTA, pH 7.5, in 12-ml polycarbonate tubes. The tubes were centrifuged at 40,000 rpm in a Beckman SW 50.1 rotor for 17 hours at 15° C. The supernatant fluid was discarded, the walls of the tubes were dried, and the pellets were resuspended in 1.25 ml of 10 mM Tris-HCl, 1 mM EDTA, 1% sodium dodecyl sulfate (SDS), pH 7.5, with 200 µg/ml of Proteinase K (Boehringer-Mannheim). After incubation at 37° C for 30 minutes, the solution was extracted 3 times with phenol. The RNA in the final aqueous phase was precipitated 3 times with ethanol and then dissolved in 1 ml of water.

Polyadenylated [poly(A)$^+$] RNA was prepared by twice passing about 2 mg of total RNA over an oligo-(dT)-cellulose column (Pharmacia Fine Chemicals) as described by Maniatis et al., supra, page 197. The poly(A)$^+$ RNA was precipitated twice with ethanol and dissolved in 200 µl of water. The yield was about 26 µg, as calculated from the optical density at 260 nm.

2.3. PREPARATION OF MEROZOITES

Merozoites of E. tenella were harvested from the ceca of 50 infected chickens (3 week old Hubbard Cross; Avian Services, Frenchtown, NJ) 5 days after infection with 50,000 of the above sporulated oocysts per bird. The ceca were removed and washed with phosphate buffered saline (PBS) for 15 minutes on a magnetic stirrer. The epithelial debris was partially removed by low speed centrifugation (50 x g), and the crude merozoites were recovered by centrifugation at 2,000 x g at 4° C for 10 minutes. The pellet was resuspended in Lysing Buffer (8.29 g/l NH_4Cl, 0.372 g/l Na_2EDTA, 1.0 g/l KCO_3, pH 7.6) and incubated on ice for 30 minutes. The merozoites were collected by centrifugation, washed once in PBS and passed over a column containing 1.0 g of spun nylon fiber (Scrub Nylon Fiber, Fenwall Laboratories, Deerfield, Illinois, U.S.A.) in a separatory funnel. The merozoites were collected by centrifugation as before and frozen on dry ice for RNA isolation, or further purified in diethylaminoethyl cellulose (DEAE, Whatman DE52, Whatman, Clifton, New Jersey, U.S.A.) for Western blot analysis.

For purification in DEAE cellulose, approximately 1 x 10^{10} merozoites were applied in PBS to a 10-ml bed volumn column and eluted with PBS. The merozoites were recovered in the first 100 ml of flow-through, essentially free of red blood cells and other cellular debris.

2.4. ISOLATION OF MEROZOITE mRNA

Frozen merozoite pellets containing 1 x 10^9 to 1 x 10^8 organisms were thawed into 10 ml of TEL/SDS buffer (0.2 M Tris HCl, 0.1 M LiCl, 25 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), pH 8.8) containing 1 mM dithiothreitol (DTT) and 300 units of RNasin (Promega Biotec, Madison, Wisconsin, U.S.A.) and homogenized with 10-12 strokes in a teflon-coated tissue homogenizer. Insoluble debris was separated by centrifugation in the cold at 3,000 x g. The supernatant fluid was extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1,v/v) which had been equilibrated with the TEL buffer.

The aqueous phase was digested with 100 µg/ml proteinase K at 37°C for 30 minutes and reextracted with an equal volume of phenol:chloroform (1:1), and the nucleic acid was precipitated with two volumes of ethanol for 1 hour on dry ice, or overnight at -20°C. The pellet, after centrifugation at 10,000 x g for one hour, was resuspended in TE (10 mM Tris, pH 7.5, 2 mM EDTA) and spun through a 4 ml CsCl cushion (5.7 M CsCl, 0.1 M EDTA) at 150,000 x g for 20 hours at 15°C. The RNA pellet was reprecipitated from 0.2 M potassium acetate with 2.5 volumes of ethanol. This total RNA was passed once over oligo-dT cellulose to enrich for poly(A)$^+$ RNA, as described by Maniatis, supra, page 197. A typical yield of 1.9 mg of total RNA from 5 x 10^9 merozoites contained approximately 20 µg of poly(A)$^+$ RNA.

2.5. SYNTHESIS OF OOCYST AND MEROZOITE cDNAs, AND INSERTION INTO PHAGE VECTORS

Double-stranded cDNA was synthesized from 6 µg of the sporulating oocyst poly (A)$^+$RNA as described by Gubler et al., Gene 25:263 (1983), using reverse transcriptase (BRL) to elongate from an oligo(dT) primer and RNase (BRL) and E. coli DNA polymerase I (New England Biolabs) to synthesize the complementary strand. The double-stranded cDNA was then blunt-ended with T4 DNA polymerase (BRL), and Eco RI linkers (GGAATTCC, Collaborative Research) were added after treatment with EcoRI methylase (New England Biolabs), following the manufacturers' protocols.

After digesting the thus prepared cDNA with EcoRI, a library was prepared in λgt11 (Stratagene Cloning Systems, San Diego, California, U.S.A.) as described by Huynh et al., in D. Glover (ed.), DNA Cloning Vol. I: A Practical Approach, 1985, IRL Press, Washington, D.C., pp. 49-78. The EcoRI cDNA fragments were ligated to EcoRI digested, dephosphorylated λgt11 arms (Stratagene Cloning Systems), and the resulting DNA was packaged into phage with the Gigapack® kit (Stratagene Cloning Systems), following the manufacturer's protocol.

The resulting library was amplified by plating on Y1088 host cells (ATCC No. 37195). The percentage of recombinants was estimated from the ratio of blue to colorless plaques on X-gal plates (Maniatis, supra, page 24) in the presence of isopropyl-β-D-thiogalactopyranoside (IPTG, Sigma Chemical Co.) to be about 90%.

Double-stranded cDNA copies of the merozoite poly(A)$^+$RNA were synthesized essentially as described above. The double-stranded cDNA used in the construction of the library contained from about 200 to 4,500 base pairs (bp), as judged by migration in denaturing gels [Bailey et al., Anal. Biochem. 70:75 (1976)].

The merozoite cDNA was methylated and ligated to EcoRI linkers as described above, except that CCGAATTCGG linkers (Collaborative Research) were used. Following digestion with EcoRI, the cDNAs were fractionated in Biogel A-50M to remove excess linker molecules and cDNAs smaller than approximately 300 bp, as described by Huynh et al., supra.

The cDNAs were ligated to λgt11 arms, and the DNA was packaged into phage as described above. The resulting library, which contained about 50,000 phage, was amplified by plating on Y1088 host cells. Plaque analysis on X-gal plates in the presence of IPTG showed about 90% recombinants.

3. IMMUNOLOGICAL SCREENING OF cDNA LIBRARIES

The λgt11 merozoite cDNA expression library was plated on Y1090 cells (ATCC No. 37197) at a density of about 10,000 plaques per 150 mm plate. Six such plates were incubated for 3.5 hours at 42°C, overlayered with nitrocellulose filters previously soaked in 10 mM IPTG to induce the expression of the β-galactosidase fusion protein, and incubated for an additional 4-5 hours to overnight at 37°C. The filters were removed from the plates and subjected to several batchwise washes with TBS (20 mM Tris, pH 8.0, 0.15 M NaCl). Non-specific protein binding sites were blocked by incubation in 20% fetal calf serum (FCS) in TBS for 2-4 hours on a rotary shaker, at 4°C.

Ascites fluid for nine monoclonal antibodies known to react with merozoite antigens (designated 7D4, 7D1, 20C6, 13A6, 20C1, 11B6, 3A5, 13A1 and 15B2) was pooled, adjusted to 20% FCS and 0.15 M NaCl in a final volume of 100 ml and applied to each of the filters in petri dishes, two filters per dish. The filters were incubated with the primary monoclonal antibody pool at 4°C overnight on a rotary shaker. Unbound antibody was removed by washing the filters 5-6 times with TBS at room temperature. Bound antibody was detected by incubating the filters with goat anti-mouse horseradish peroxidase (HPOD) conjugate (Boehringer-Mannheim), followed by color development using 3 mg/ml 4-chloro-1-naphthol (Bio Rad) and 0.018% H_2O_2, as described by Hawkes et al., Anal. Biochem. 119:142 (1982).

Positive plaques identified in the initial high density screen were plaque-purified in a secondary screen

using the same monoclonal antibody pool. Each positive was assigned to an individual monoclonal from the pool by plating the positives in multiple grid arrays, each of which was induced with IPTG, transferred to nitrocellulose and incubated with one of the monoclonals from the pool. One positive phage designated λm2-4 was identified which was recognized by three of the eight antibodies in the pool, antibodies 7D1, 7D4 and 20C6.

Similar methods were used to screen the sporulating oocyst cDNA library, except that a pool of monoclonal antibodies containing antibodies 6A5, 7B2, 15A3 and 20C6 was used for the initial screening; 7B2, 15A3, 20C6 and 8A2 were used in a second screening; and 15A3, 7B2 and 20C6 were used in a third screening; and the incubation buffer contained in addition 0.05% Tween-20 [polyoxyethylene(20) sorbitan monolaurate]. In this way, plaques designated λS1-3, λS1-4 and λS1-7; λS2-1, λS2-4 and λS2-5; and λS3-1 which were recognized by monoclonal antibodies 6A5, 8A2 and 7B2, respectively, were identified in the oocyst cDNA library. DNA made from the phage producing protein that reacted with the 6A5 antibody was analyzed by digestion with EcoRI and electrophoresis in agarose gels (Maniatis et al., supra, page 150). Three different inserts having sizes of approximately 1150, 890 and 615 bp were thus found.

4. EXPRESSION OF EIMERIA GENES IN E. COLI

The 1.1 kb and 0.9 kb EcoRI DNA molecules from phages λS1-7 and λS1-3, respectively, were isolated and inserted into the EcoRI site of each of three variable reading frame expression vectors, pEV-vrf1, pEV-vrf2 and pEV-vrf3, constructed as described by Crowl et al., Gene 38:31 (1985). Plasmids containing the inserts in both possible orientations were transformed as described by Mandel et al. [J. Mol. Biol. 53:159 (1970)] into E. coli strain MC1061 carrying the compatible plasmid pRK248clts described by Bernard et al. [Methods in Enzymology 68:482 (1979)]. Strain MC1061 and plasmid pRK248clts have been deposited with the American Type culture Collection and assigned accession Nos. ATCC 53338 and 33766, respectively.

The bacterial transformants were grown at 30° C in M9 medium [Maniatis et al., supra, page 68] with 0.5% glucose and 0.5% Casamino acids and shifted to 42° C at an O.D. (550 mμ) of 0.5 as described by Crowl et al., supra, to induce transcription at the λP_L promoter. After incubating for 2-3 hours, 1-ml samples were taken, and the cells in the samples were collected by centrifugation. The cell pellets were treated as described by Crowl et al., supra, and the lysates were subjected to sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis as described by Laemmli, Nature 227:680 (1970). Following electrophoresis, the proteins in the gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes for Western blot analysis [Towbin et al., Proc. Natl. Acad. Sci. USA 76:4350 (1979); Burnetti, Anal. Biochem. 112: 195 (1981)], using the 6A5 monoclonal antibody and goat anti-mouse HPOD conjugate for detection.

This analysis showed that the 0.9 kb cDNA molecule in one orientation in vector pEV-vrf1 produced a 20 kd protein that reacted with the 6A5 antibody. No expression was observed with the 1.1 kb molecule, probably because it contained 5′ noncoding sequences. To optimize the yield of this protein, various expression media were examined. It was found that the preferred medium contained per liter (± 10%) 6.0 g KH_2PO_4, 4.0 g K_2HPO_4, 5.0 g $(NH_4)_2SO_4$, 3.5 g $MgSO_4$-$7H_2O$, 21.0 g Yeast Extract, 3.5 g Bacto Tryptone, 1.0 ml LB625 Antifoam, 25 g glucose, 70 mg Thiamine-HCl, 2.5 ml vitamin solution [GIBCO MEM (100X) Vitamin Solution] and 1.0 ml trace elements. LB625 Antifoam, a product of Union Carbide, is a linear polymer of ethylene and polypropylene oxide having a viscosity of 625 Saybolt Universal Seconds at 37.8° C.

Vitamins per liter of fermentation broth included 0.25 mg each of D-Ca pantothenate, choline chloride, folic acid, nicotinimide, pyridoxal-HCl and additional thiamine-HCl; 0.50 mg of i-inositol; and 0.025 mg of riboflavin. Trace elements per liter of broth included 2.7 mg of
$FeCl_3$-$6H_2O$; 0.8 mg each of $ZnSO_4$-$7H_2O$ and
$CuSO_4$-$5H_2O$; 0.7 mg each of $CoCl_2$-$6H_2O$ and
Na_2MoO_4-$2H_2O$; 0.2 mg of H_3BO_3; and 0.5 mg of $MnSO_4$-H_2O.

The nature of the immunoreactive protein expressed by phage λm2-4 was investigated first in a lysogen isolated from an infection of Y1090 cells by differential growth at the permissive (30° C) and non-permissive (42° C) temperatures. For Western blot analysis of the proteins synthesized by this lysogen, a 50 ml culture was grown at 30° C in LB medium [Maniatis et al., supra, page 69] to an O.D. (550 mμ) of 0.5, and shifted to 42° C to induce replication of the phage. After 15 minutes at 42° C, IPTG was added to 10 mM, and incubation was continued at 37° C for 30 minutes. The cells were harvested by centrifugation at 4° C and lysed by boiling for 5 minutes in Laemmli sample buffer (0.125 M Tris, pH 6.8, 1% (w/v) SDS, 1.4 M β-mercaptoethanol, 001% bromophenol blue (w/v), 20% (v/v) glycerol).

The equivalent of 1.0 ml of culture was resolved by electrophoresis in a 12.5% SDS-polyacrylamide gel, transferred electrophoretically to nitrocellulose and probed as described above with a pool of the three monoclonal antibodies (7D1, 7D4 and 20C6) which identified the λm2-4 phage. Development of the Western blot revealed a fusion protein of greater than 150 kd size which was present in the induced lysogen (Figure 10, panel B, Lane 2). Antibody specific for β-galactosidase also reacted with this high molecular weight protein, and to a protein of approximately 114 kd, the expected size of β-galactosidase alone (see Fig. 10, panel A, lane 2).

The phage λm2-4 DNA was digested with EcoRI to produce a1.7 kb DNA molecule. This molecule was subcloned into an EcoRI-linearized plasmid pool containing plasmids pEV-VRF1, 2 and 3 [Crowl et al. supra] and transformed into E. coli strain MC1061 containing plasmid pRK248clts, as described above. Transformants were screened for expression of an immunoreactive protein upon temperature induction, using the pool of three monoclonal antibodies shown to react with the fusion protein in the λm2-4 lysogen. The immunoreactive recombinant protein was further characterized by Western blot analysis of the E. coli lysates, employing one of the three monoclonal antibodies, 7D4, from the pool. Each of the positive colonies was found to contain plasmid DNA with the expected 1.7 kb insert, and to direct the synthesis of a protein of approximately 65 kd upon induction, as determined by SDS-polyacrylamide gel electrophoretic analysis.

The expression of this 65 kd protein was found to be relatively insensitive to variations in growth medium and induction protocol. The protein was recovered quantitatively in the supernatant following sonic disruption of the cell pellet.

The expression plasmid containing the 1.7 kb insert was used in the subsequent production of recombinant protein and is shown schematically in Figure 11. This plasmid was propagated in MC 1061 host cells lysogenic for λcI857 (prepared using standard methods for the generation of λ phage lysogens described by Arber et al., in Cold Spring Harbor Monograph, Lambda II, 1983, Hendrix et al., Eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, p. 450) at 30° C, to maintain the repressed state of the P_L promoter in the plasmid.

Using similar methods, expression of a 28 kd protein encoded by a sporulating oocyst cDNA segment having about 1.1 kb was carried out. This protein bound specifically to monoclonal antibody 8A2. Expression of a 45 kd protein encoded by a sporulating oocyst cDNA of about 1.2 kb was also carried out. This protein bound specifically to monoclonal antibody 7B2.

5. DNA SEQUENCE ANALYSIS

In general, small scale isolation of plasmid DNA from 1 ml of saturated overnight cultures was carried out using the procedure of Birnboim et al. [Nucleic Acids Research 7:1513 (1979)]. This procedure allows the isolation of a small quantity of DNA from a bacterial colony for analytical purposes. Larger amounts of plasmid DNA were prepared using 1-liter cultures following a standard protocol with cesium chloride centrifugation. [Maniatis et al., supra, page 93].

The DNA sequences of the cDNAs from the sporulating oocyst library were determined by the chemical cleavage method of Maxam et al., Methods in Enzymology 65:499 (1980) and by the chain-termination method of Sanger et al., Proc. Natl. Acad. Sci. USA 74:5463 (1977), as modified for double-stranded plasmid DNA by Smith et al., Cell 16:753 (1979) and Wallace et al., Gene 16:21 (1981). In the chain termination protocol, 7-deaza-dGTP [Barr et al., BioTechniques 4:428 (1986)] was substituted for dGTP to eliminate G-C compression artifacts.

To facilitate sequence analysis, the 1.1 kb EcoRI molecule from λS1-7 was transferred to plasmid pEV3-SEQ (Fig. 12), which has a polylinker next to the EcoRI site of pEV-vrf3. This polylinker was used to linearize the plasmid at the BamHI and KpnI sites to generate unidirectional deletions with exonuclease III [Henikoff, Gene 28:351 (1984)]. The XbaI site in the polylinker was used for 3′-end labeling for Maxam-Gilbert sequencing of the deletions, and the primer CGGTCGACTCGAGCCA was used for Sanger sequencing. This primer was ^{32}P labeled at its 5′ end using [γ-^{32}P]ATP (ICN) and polynucleotide kinase as described by Maniatis et al., supra, page 122.

Fig. 13 shows the restriction sites in the 1.1 kb EcoRI molecule used for Maxam-Gilbert sequencing. The position of the EcoRI sites in the 0.9 kb molecule are also shown, since these were also used. The end points of deletions made with exonuclease III are also shown. These were sequenced either from the XbaI site in the pEV3-SEQ polylinker by the Maxam-Gilbert method or with a primer extension (Fig. 12) by the Sanger method. Both stands of the entire cDNA were sequenced by one or both of these methods. Due to

a high G-C content in the DNA, the Maxam-Gilbert reactions were usually fractionated in both 8% and 15% polyacrylamide-urea gels.

Primer extension was carried out by incubating 1.5 µg of poly(A)$^+$ RNA with 2 pmoles of the 5′-end labeled synthetic oligonucleotide primer, GAGGTCTGCCATTTTGC, for 60 minutes at 42° C in 50 mM Tris-HCl, pH 8.0, 8 mM $MgSO_4$, 0.1 M NaCl, 2 mM dithiothreitol, 2 mM of each deoxynucleotide triphosphate (dNTP, Pharmacia Fine Chemicals), 20 units RNasin (Promega Biotec, Madison, WI) and 20 units AMV reverse transcriptase (Pharmacia, Piscataway, NJ, FPLCpure). The products were analyzed in the 8% polyacrylamide-urea gels used for sequence analysis, with ^{32}p-labeled HpaII digested pBR322 DNA as a molecular size marker.

For sequence analysis, the primary products were eluted from the gel and analyzed by the chemical cleavage method of Maxam et al., supra, or ddNTPs were used in the extension reaction [Tolan et al., J. Biol. Chem. 259:1127 (1984); Graves et al., J. Biol. Chem. 261:11409 (1986)]. The reactions were analyzed in 8% polyacrylamide-urea gels.

The nucleotide sequence of the 1.1 kb cDNA molecule is shown in Fig. 14. The sequence of the 0.9 kb molecule extends from base 188 to base 1082 within this larger molecule. The amino acid sequence predicted from open reading frame analysis of this nucleotide sequence is shown in Fig. 15. The correctness of the predicted amino acid sequence shown in Fig. 15 was confirmed as follows.

Synthetic polypeptides were prepared having amino acid sequences corresponding to residues 41-54 and 145-164 of Fig. 15. Rabbit antisera raised against both of these polypeptides were used in Western blot analysis of both total sporozoite proteins and a lysate of the E. coli transformant expressing the 0.9 kb cDNA. The antibodies against both of the polypeptides bound to proteins in both of the Western blots.

Using similar methods, the nucleotide sequence of the 1.7 kb insert encoding the 65 kd protein in phage λm2-4 was determined, with the results shown in Fig. 16. The predicted amino acid sequence of the protein encoded by this DNA sequence is shown in Fig. 17. This sequence was confirmed by amino acid sequence analysis performed on polypeptides produced by tryptic digestion of the expressed 65 kd protein, as described below. Regions in the overall amino acid sequence corresponding to some of these peptides are underlined in Fig. 17.

Curiously, the DNA sequence open reading frame for the 1.7 kb molecule would be expected to encode a protein of about 33,349 daltons. Yet, the expression product from this DNA fragment migrates in SDS gels with an apparent molecular weight of about 65 kd. The reason for this discrepancy between the predicted and observed protein size is unknown. This protein is referred to herein as the "65 kd" protein.

In a similar fashion, the nucleotide and predicted amino acid sequences of the cDNA molecule encoding the 28 kd protein recognized by monoclonal antibody 8A2 were determined, with the results shown in Figs. 18 and 19, respectively.

Similarly, the nucleotide and predicted amino acid sequence of the 1.2 kb 782 cDNA were determined. Since a continuous open reading frame was found and the protein isolated from sporozoites by immunoprecipitation is larger than 200 kd, the library was screened for larger cDNAs, using the 1.2 kb cDNA as a probe. A3.2 kb cDNA was thus obtained, having the nucleotide and predicted amino acid sequences shown in Figs 20 and 21, respectively.

6. PURIFICATION AND CHARACTERIZATION OF THE 65 KD PROTEIN

6.1. PROTEIN PURIFICATION

High cell density fermentation of E. coli MC1061 (pRK248clts) containing the pEV/2-4 expression plasmid was carried out in 10-liter fermenters in 1.5 x M-9 medium, using standard protocols of temperature induction as described above after approximately 4 hours of growth at the permissive temperature. The cell mass was harvested 5 hours after induction, yielding 500 grams of cell paste.

Fifty grams of the E. coli cell paste were uniformly suspended in 500 ml of 10 mM Tris-HCl, 5 mM EDTA, pH 8.0, and stirred at 2-8° C for two hours. The suspension was passed through a Gaulin homogenizer (APV Gaulin, Everett, Massachusetts, U.S.A.) two to three times at 7,000 psi. The cell lysate was centrifuged at 24,000 x g for one hour in a Sorvall RC-5 centrifuge, and the pellet was discarded. Solid ammonium sulfate was added to the supernatant (final concentration 80%). This was kept at 4° C for two hours, and then centrifuged at 24,000 x g for one hour. The pellet was dissolved in 20 mM potassium phosphate, pH 6.8. After centrifugation, the supernatant was dialyzed against 20 mM potassium phosphate,

pH 6.8.

A Pharmacia glass column (5 cm diameter x 10 cm length) was packed with NuGel P-DE 200® (200 Angstrom, 40-60 µm, weak anion exchange, Separation Industries, Metuchen, NJ) silica support. The gel was equilibrated with 20 mM potassium phosphate, pH 6.8. The sample was loaded (10 ml/min), washed with equilibration buffer and eluted with 20 mM potassium phosphate containing 0.4M NaCl, pH 6.8. The column fractions were analyzed by Western blotting with antibody 7D4 to detect the 65 kd protein.

An immunoaffinity column was used to further purify the 65 kd protein. The adsorbent for this column was prepared by immobilizing monoclonal antibody 7D4 on NuGel P-polyaldehyde® (500 Angstrom, 40-60 µm, Separation Industries, Metuchen, New Jersey, U.S.A.) silica support. The immobilization procedure involved the following: 10 grams of polyaldehyde support were suspended and washed with 0.1M potassium phosphate, 0.1M NaCl, pH 6.8, and transferred quantitatively into an Ehrlenmeyer flask containing 20 ml of monoclonal antibody 7D4 at a protein concentration of 8 mg/ml. Sodium cyanoborohydride (4 mg) was then added to the suspension. The mixture was shaken gently at 4° C for 16 hours. The gel was filtered and washed with 0.1M potassium phosphate, 0.1M NaCl, pH 6.8. Pooled filtrates were checked for unbound antibody. Binding density was 8 mg/g of support. Uncoupled activated sites were blocked by suspending the gel in 20 ml of 1M ethanolamine, pH 7.5 Sodium cyanoborohydride (4 mg) was added to the suspension, which was then agitated at 4° C for 16 hours. The gel was collected and washed thoroughly with cold coupling buffer.

To carry out the immunoaffinity chromatography, a column (1 cm x 10 cm) was packed with the immobilized 7D4 antibody and equilibrated with cold phosphate buffered saline (PBS) containing 0.1% Triton x-100. A pool of fractions from the NuGel P-DE 200® column containing the 65 kd protein was diluted 2 x with PBS containing 0.1% Triton X-100 and loaded onto the column at a flow rate of 10 ml/min. After loading, the gel was washed with PBS to remove unadsorbed material. The adsorbed immunoreactive material was eluted with 0.3 M acetic acid, 0.1 M NaCl, pH 2.7, buffer. The protein was then concentrated in an Amicon Stircell® apparatus using a YM 10 membrane (Amicon, Div. W.R. Grace & Co. Danvers, Massachusetts, U.S.A.).

The purity of the protein was determined by SDS polyacrylamide gel electrophoresis as described by Laemmli [Nature 227:680 (1970)]. The gel was stained with Coomassie blue. Western blot analysis was also carried out, using the 7D4 monoclonal antibody with goat anti-mouse horseradish peroxidase conjugate. The results are shown in Fig. 22. Lanes 2, 3, 4 and 5 contain purified protein from two preparations. Lanes 1 and 6 contain a mixture of molecular weight marker proteins having the molecular weights shown to the left and right of the figure. Ten micrograms of protein were run in each lane.

In Fig. 22, it can be seen that the purified protein migrated in the SDS gel as a major band having an apparent molecular weight of about 65 kd, with minor bands having higher and lower mobility.

6.2. ISOELECTRIC POINT DETERMINATION

Ten micrograms of the purified 65 kd protein were subjected to isoelectric focusing in a preformed isoelectric focusing gel obtained from LKB Instruments, Gaithersburg, Maryland, U.S.A.. A mixture of standard proteins having known isoelectric points was run at the same time. The gel was run for about 2 hours at 50 mA, 1,500 V according to the manufacturer's instructions, using a 3.5-9.5 pH gradient.

Upon completion of electrofocusing, the gel was stained with Coomassie blue dye to detect the protein bands. The isoelectric point of the purified protein was then determined by measuring the position of the band within the pH gradient in relation to the positions of the protein standards. The isoelectric point of the protein thus determined was 4.6.

6.3. AMINO ACID COMPOSITION ANALYSIS

Amino acid composition analysis was carried out using post column reaction with fluorescamine as described by Pan et al., in Methods of Protein Microcharacterization, 1986, Shively, ed., The Humana Press, pp. 105-119. Samples containing 3 µg of the 65 kd protein were hydrolyzed in 6 N HCl containing 4% thioglycolic acid at 110° C for 20 to 24 hours in vacuo, and 10% of the hydrolysate was used for analysis. Cysteine values were determined after performic acid oxidation. The results are shown in Table 3.

TABLE 3

AMINO ACID COMPOSITION ANALYSIS OF THE 65 KD PROTEIN	
AMINO ACID	MOLE PERCENT
Asp	6.06
Thr	6.07
Ser	7.27
Glu	18.24
Pro	5.35
Gly	16.76
Ala	11.71
Cys	4.45
Val	4.88
Met	2.08
Ile	2.17
Leu	3.22
Tyr	2.20
Phe	2.13
His	1.07
Lys	2.72
Arg	3.61
Trp	ND
ND = Not Dererminded	

6.4. N- AND C-TERMINAL SEQUENCE ANALYSIS

Two hundred picomoles of the protein (as determined by amino acid composition analysis) were subjected to N-terminal analysis, using the method of Hewick et al.; J. Biol. Chem. 256:7990 (1981) and an Applied Biosystems model 470A sequencer (Applied Biosystems, Inc., Foster City, California, U.S.A.). The N-Terminal sequence thus determined was M-N-K-N-S-?-L-G-G-F-?-S-M-Q-E-S-P-P-P-. The identities of the amino acid residues at positions indicated by question marks are uncertain because the recovery of PTH-Cys was low, and cysteine residues might be involved in disulfide linkages [Hewick et al., J. Biol. Chem. 256:7990 (1981)].

C-Terminal analysis was performed on 1200 picomoles of the 65 kd protein by time course carboxypeptidase Y digestion as described by Hayashi, Methods in Enzymology 47:84 (1977). Carboxypeptidase Y (Boehringer Mannheim, Indianapolis, IN) was used at a concentration of 0.8 µg/350 µl in 0.05 M sodium acetate buffer, pH 5.9, and sample aliquots were taken after 0, 2, 5, 10, 20 and 30 minutes for analysis. The sample aliquots were acidified with HCl to stop further reaction and then subjected to amino acid analysis as described above. This analysis showed that the amino acid sequence at the C-terminus is probably (Met, Trp)-Ala-Ser. Tryptophan was observed to increase simultaneously with the methionine, but Trp is difficult to quantify in the fluorescamine analyzer because it has a low reactivity with fluorescamine. Therefore, the relative positions of Trp and Met could not be determined with certainty by this analysis.

6.5. TRYPTIC PEPTIDE ANALYSIS

In part to verify the amino acid sequence predicted from the nucleotide sequence of the cDNA encoding the 65 kd protein, some of the protein was digested with trypsin (Cooper Biomedical, Philadelphia, Pennsylvania, U.S.A.) and the resulting peptides were sequenced as described below.

Tryptic digestions were carried out overnight at 37° C on 148 µg of protein in 0.2M ammonium bicarbonate, pH 8, using an enzyme-to-substrate ratio of 1:30 (weight or molar basis?). Peptides thus generated were separated in a Waters HPLC system using an Altex ultrasphere 250 x4.6 mm C-18 column (Beckman Instruments, Fullerton, CA) with a 0 to 55% gradient of increasing acetonitrile in 0.1% (basis) trifluoroacetic acid. Prior to the HPLC separation, the digest was reduced with β-mercaptoethanol for 30 minutes at 37° C to break any disulfide bonds in the peptides. Column effluent was monitored at 215 mµ using a laboratory Data Control detector (Laboratory Data Control, Rivera Beach, Florida, U.S.A.). The HPLC column resolved 8 major peaks, as shown in Fig. 23A.

Each peak was first analyzed by amino acid analysis as described above to determine both the quantity and the composition of the peptides. Then, most of the peptide peaks from the HPLC column were sequenced by automated Edman degradation, using an Applied Biosystems Model 470A gas phase sequencer. Phenylthiohydantoin (PTH) amino acid derivatives were identified in a Waters HPLC system using an Altex ultrasphere C-18 column as described by Hawke et al. [Anal. Biochem. 120:302 (1982)], or in an Applied Biosystems Model 120A on-line PTH amino acid analyzer.

The amino acid sequences of some of these peptides are shown under the underlined regions of Fig. 17. The number of each of these peptides (corresponding to the HPLC peak numbers) is shown circled beside the corresponding sequence. Uncertainly in the identity of some of the residues in the peptide sequences is indicated by a question mark at those positions, although the amino acid composition analyses of the peptides showed that the amino acids indicated in the corresponding positions of the predicted sequence were present in the peptides. The uncertainly in the identity of some of the peptide residues was due to the low reactivity of tryptophan with fluorescamine.

Peptide 6, which corresponds to the N-terminus of the complete 65 kd protein, contains 4 residues at its N-terminus which are encoded by nucleotides in the expression plasmid. Analysis of peptide 8 produced an amino acid sequence similar to that of peptide 3 but lacking the 4 C-terminal residues, suggesting that it was probably the result of incomplete tryptic digestion. Peptide 5 was not sequenced, because amino acid composition analysis of this peptide showed that it was the same as peptide 6, less the first 3 amino acid residues at the N-terminus.

HPLC analysis of the tryptic digest carried out as described above but without prior mercaptoethanol reduction produced an elution profile in which peaks 4, 7 and 8 were absent (Fig. 23 B). This observation suggests that these cysteine-containing peptides were probably involved in disulfide bond formation in the unreduced protein.

7. POULTRY IMMUNIZATION

7.1. USE OF THE 65 Kd ANTIGEN

To determine whether administration of the purified recombinant 65 kd protein could protect chickens against challenge by Eimeria tenella sporulated oocysts, a series of immunization experiments was performed. In these experiments, one day to three week old Leghorn chickens (Avian Services, Frenchtown, New Jersey, U.S.A.) were maintained in a clean room and cared for by attendants who did not have contact with other birds up to the time of challenge. The birds were kept in electrically heated brooder cages until they were 3 or 4 weeks old, after which they were transferred to grow-out cages.

Non-medicated broiler starter feed and water were supplied ad libitum throughout the experiments. At the time of challenge with oocysts, the birds were transferred to another building where they were kept until the end of the experiments. The clinical conditions of the animals were checked at least three times weekly before immunization and on a daily basis after immunization. The birds were individually identified by means of wing bands at 3 or 4 weeks of age before random assignment into various test groups.

Various lots of the 65 kd protein purified by immunoaffinity chromatography as described above were used as the immunogen. These lots of immunogen contained bacterial endotoxin activity ranging from about 0.3 to about 50 endotoxin units per µg of protein, with activity determined and defined as described in the United States Pharmacopeia, 21st Revision, 1985, United States Pharmacopeial Convention, Inc., Rockville, Md., pp. 1165-1167. The protein was dissolved in 0.02 M K_2HPO_4 buffer, pH 6.8, before use and diluted with the same buffer as required.

Bovine serum albumin (BSA, Pentex) was used as a control. Because pyrogenic activity was present in

the immunogen used, approximately equal amounts of such activity were added to all BSA controls, to account for any nonspecific effects that might be due to this activity. This pyrogenic activity was added to the BSA in the form of an untransformed E. coli lysate which was prepared by disrupting E. coli by sonication and then filtering the material through a 0.45 μ Millipore filter.

Diluted samples of control BSA or the Eimeria antigen were combined with an equal volume of adjuvants and mixed thoroughly in glass syringes fitted with 18 gauge needles prior to administration. Freund's complete and incomplete adjuvant were used for primary and booster immunizations, respectively. Both adjuvants were obtained from GIBCO, Grand Island, New York, U.S.A..

Primary immunizations were made subcutaneously on the posterior portion of the body at the base of the neck, when the birds were 4 weeks old. Some birds also received booster immunizations at 6 weeks of age. The volume of injected material ranged from about 0.4 to 2.4 ml. For the larger volumes, the dose was divided between 2 injections. Two or three weeks after the last vaccination, birds were challenged with 25,000 or 50,000 sporulated oocysts of E. tenella, administered orally. Seven days post infection, the surviving birds were sacrificed, necropsied and scored for gross lesions. All birds that died during the experiments were also necropsied. Diagnoses were made, and the intestinal lesions were scored as 0 = normal, 1 = slight infestation, 2 = moderate infestation, 3 = severe infestation and 4 = death. The readings obtained were summarized as the average degree of infection for each group of birds. The birds were also weighed at the time of infection and 7 days post infection. Some birds were not vaccinated with BSA or coccidial antigen but were treated as infected or uninfected, unvaccinated controls.

The results of two such experiments are shown in Table 4.

TABLE 4

EFFECT OF SUBCUTANEOUS IMMUNIZATION OF CHICKS GIVEN ONE OR TWO VACCINATIONS WITH PURIFIED RECOMBINANT 65 KD ANTIGEN

No. Birds	Treatment[a]	Dose (µg) at Age 4 Weeks	Dose (µg) at Age 6 Weeks	Lesion Score[b]	Weight Gain/Loss[c] (grams)
			Experiment 1		
10	IUC	-	-	2.8	-25
8	Antigen	3.15	-	2.4	-44
10	Antigen	12.25	-	2.5	-10
6	BSA	17.5	-	3.0	+40
10	UUC	-	-	0	+107
10	IUC	-	-	2.9	-40
8	Antigen	3.15	1.6	2.0[e]	-15
10	Antigen	17.5	13.2	1.8[e]	+5
8	BSA	12.25	13.2	2.5	-13
10	UUC	-	-	0	+87
			Experiment 2		
8[d]	IUC	-	-	2.6	-11
10	Antigen	4	-	2.2	+65
10	Antigen	20	-	2.0	+19
9	Antigen	100	-	2.9	+14
10	BSA	100	-	2.4	-7
10	IUC	-	-	2.5	+15
10	Antigen	4	4	2.1	+35
10	Antigen	20	20	2.0	+74
8	Antigen	100	100	2.1	+81
10	BSA	100	100	2.4	+69
4	UUC	-	-	0	-3

a- IUC, Antigen, BSA and UUC refer to infected (with oocysts) unimmunized controls, purified 65 kd protein, bovine serum albumin and uninfected unimmunized controls, respectively.

b- In Experiment 1, birds given a single immunization were challenged 3 weeks later with 50,000 sporulated oocysts of E. tenella; those given a booster vaccination were challenged 2 weeks after that with 25,000 of the oocysts. In Experiment 2, the timing of oocyst challenges was the same, but 25,000 oocysts were given to both singly vaccinated and boosted birds. Infected unimmunized controls were maintained for each experiment and given identical numbers of sporulated oocysts at the same age, seven days prior to sacrifice. Results are based on a score of 0-4, as described in the text.

c- Values shown are the difference between weight at time of infection and weight 7 days post infection.

d- This group originally contained 9 birds, but one died after 1 week.

e- $P \leq 0.05$ compared to IUC.

The data of Table 4 show that vaccination with the 65 kd protein generally produced numerically lower lesion scores, compared to infected but unimmunized controls. Two groups of birds given booster vaccinations in Experiment 1 (denoted by superscript e in the Table) showed reduced lesion scores that were statistically significant. In other cases, the degree of reduction in lesion scores was not as great, but weight gain was nevertheless generally improved in the vaccinated birds.

To determine whether a third vaccination would further enhance protection, an experiment was carried out in which groups of 8 birds were treated as infected or uninfected, unvaccinated controls or vaccinated with BSA or the merozoite protein at 3 and 5 weeks of age or at 3, 5 and 7 weeks of age. The first two vaccinations were made with Freund's complete adjuvant. Where a third vaccination was given, it was given with Freund's incomplete adjuvant. Inoculations were given subcutaneously as described above.

Two weeks after the last vaccination, each bird was challenged with 25,000 sporulated oocysts of E. tenella by the oral route. Body weights were measured at the time of challenge and 7 days thereafter, at which time the birds were sacrificed and cecal lesions were scored. The results are shown in Table 5.

TABLE 5

EFFECT OF SUBCUTANEOUS IMMUNIZATION OF CHICKS GIVEN TWO OR THREE VACCINATIONS WITH PURIFIED RECOMBINANT 65 KD ANTIGEN

Treatment[a]	Dose (µg) at Age			Lesion Score[b]	Weight Gain/Loss[c] (grams)
	3 Weeks	5 Weeks	7 Weeks		
IUC	-	-	-	2.13	+59
Antigen	4	4	-	1.75	+122
Antigen	4	4	-	2.88	+128
Antigen	20	20	-	1.88	+87
Antigen	20	20	-	1.88	+69
BSA	20	20	-	3.13	+106
BSA	20	20	-	2.38	+131
UUC	-	-	-	0	+131
IUC	-	-	-	2.25	+23
Antigen	4	4	4	2.25	+91
Antigen	20	20	20	2.25	+86
BSA	20	20	20	1.75	+78

a- IUC, Antigen, BSA and UUC refer to infected (with oocysts) unimmunized controls, purified 65 kd protein, bovine serum albumin and uninfected unimmunized controls, respectively.

b- Birds were challenged 2 weeks after the last vaccination with 25,000 E. tenella sporulated oocysts, or seven days prior to sacrifice for infected unimmunized controls. These controls were seven and nine weeks old for double and riple immunization studies, respectively, when infected. Results are based on a score of 0-4, as described in the text.

c- Values shown are the difference between weight at time of infection and weight 7 days post infection.

The data in Table 5 show that immunity was not improved by administering a third vaccination. Greater protection, as shown by reduced cecal lesion scores, was conferred by the antigen, compared to untreated infected controls or birds vaccinated with BSA.

To determine whether routes of administration other than subcutaneous injection might produce better results, two dosage levels of the 65 kd protein were administered three times, two weeks apart, to groups of 8 3-week-old Leghorn chicks, using intradermal, subcutaneous, intramuscular, oral and anal routes of administration. Two weeks after the last immunogen administration, the birds were challenged with 25,000 sporulated oocysts of Eimeria tenella given orally. The birds were sacrificed one week after challenge, and cecal lesion scores were determined.

Subcutaneous injections were administered as described above. Intramuscular injections were made deeply into the exterior side of the left thigh. Intradermal injections were administered into the anterior side of the right wing. Oral administration was delivered using a 5 cm long 18 gauge ball-tipped needle, depositing the inoculum into the crop of the bird. Anal administration was made using a 5 cm long 18 gauge olive-tipped needle, introduced to its maximum length into the cloacal opening. After oral and anal administration, the birds were held in standing and inverted positions, respectively, for several minutes, to avoid possible expulsion of the inoculum.

The subcutaneous dosage form was as described above, with Freund's complete adjuvant used for the primary injection and Freund's incomplete adjuvant used for the booster injections. Dosage forms for the other routes of administration contained protein at the indicated levels in 0.02 M K_2HPO_4 buffer, pH 6.8.

The results of this experiment are shown in Table 6.

TABLE 6

EFFECT OF VARIOUS ROUTES OF ADMINISTRATION ON CHICK VACCINATION WITH THE 65 KD ANTIGEN

Treatment/Route[a]	Dose (mg) at Age			Lesion Score[b]	Weight Gain/Loss[c] (grams)
	3 Weeks	5 Weeks	7 Weeks		
IUC	-	-	-	2.9	+58
Antigen/SC	5	5	5	2.3	+66
Antigen/SC	25	25	25	2.8	+68
BSA/SC	25	25	25	2.8	+47
Antigen/IM	5	5	5	2.3	+33
Antigen/IM	25	25	25	2.3	+72
Antigen/A	5	5	5	2.5	+53
Antigen/A	25	25	25	2.6	+50
Antigen/O	5	5	5	1.8[d]	+67
Antigen/O	25	25	25	2.5	+87
Antigen/ID	5	5	5	2.1	+26
Antigen/ID	25	25	25	1.9[d]	+114
UUC	-	-	-	0	+114

a- IUC, Antigen, BSA and UUC refer to infected (with oocysts) unimmunized controls, purified 65 kd protein, bovine serum albumin and uninfected unimmunized controls, respectively. SC, IM, A, O and ID refer to subcutaneous, intramus ular, anal, oral and intradermal routes of administration, respectively.

b- Birds were challenged 2 weeks after the last vaccination with 25,000 E. tenella sporulated oocysts, or seven days prior to sacrifice for infected unimmunized controls. These controls were 9 weeks old when infected. Results are b sed on a score of 0-4, as described in the test.

c- Values shown are the difference between weight at time of infection and weight 7 days post infection.

d- $P < 0.05$ compared to IUC.

Table 6 shows that the lowest cecal lesion scores were observed in birds immunized with 5 µg of antigen by the oral route and with 25 µg of antigen by the intradermal route. These results were statistically significant. Numerically lower lesion scores were seen for other routes of administration and other dosage levels, indicating a protective trend. The differences between these scores and those of the IUC birds, however, were not statistically significant.

Failure to observe linear dose responses in the foregoing experiments may have been due to differences in trace contaminants and/or pyrogenic content in the 65 kd antigen preparations, or to other factors.

7.2. VACCINIA VECTOR VACCINATION

To produce a more effective means of immunizing chicks with the E. tenella antigens of this invention, the 1.1 kb cDNA encoding the 20 kd protein recognized by monoclonal antibody 6A5 (Fig. 14) and the 1.1 kb cDNA molecule encoding the 28 kd protein recognized by monoclonal antibody 8A2 (Fig. 18) were cloned into vaccinia virus and used to vaccinate chicks, as described below.

7.2.1. VECTOR PREPARATION

All recombinants made were based on homologous recombination into the viral thymidine kinase (TK) locus as described by Mackett et al. [Proc. Natl. Acad. Sci. USA 79:7415 (1982)]. The TK locus has been mapped to the vaccinia virus (VV) HindIII J fragment [Hruby et al., J. Virol. 43:403 (1982)], and part of this fragment has been sequenced [Weir et al., J. Virol. 46:530 (1983)].

To construct a vector for recombination, the VV HindIII J fragment was subcloned into pUC8 (Fig. 24a). This construct was cleaved with HpaII. The fragments were treated with E. coli DNA polymerase Klenow fragment (Klenow) and recleaved with HindIII, and the piece containing the viral TK gene was isolated from low melting agarose. The isolated fragment was ligated into the HindIII and blunt ended (S1 treatment) EcoRI site of a pUC8 vector (Fig. 24b, right). Subsequently, the HindIII site was eliminated by treating the HindIII digested DNA with Klenow and religating the vector fragment. For the insertion of the VV promoter (designated the 7.5K promoter), the vector was cleaved by ClaI and EcoRI.

The VV 7.5K promoter is located in one of the smallest SalI fragments of the virus [Venkatesan et al., Cell 25:805 (1981)]. The corresponding fragment was cloned into MI3mp8. A clone was selected in which the direction of transcription was toward the EcoRI site of MI3mp8 (Fig. 24a, left). The DNA was cleaved with ScaI and SmaI, BglII linkers were added and the DNA was religated (Fig. 24b). The EcoRI-AccI fragment containing the viral promoter segment was isolated from the MI3 construct and ligated into the pUC8-TK fragment described above resulting in vector pUC8-TK-7.5K*. This new vector was digested with BglII and EcoRI.

To create a vector with multiple cloning sites, an appropriate polylinker was included in the above construct. For this purpose the polylinker contained in the MI3tgI3I (Amersham) was chosen (Fig. 24d). The polylinker fragment was isolated by digesting the phage DNA with BglII and EcoRI, and the fragment was inserted into the pUC8-TK-7.5K* construct, resulting in the final basic vector for recombination of foreign antigens into VV (Fig. 24c) which is pUC8-TK-7.5K.

The EcoRI fragment coding for the 28 kd protein which binds to monoclonal antibody 8A2 does not contain the sequence for the N-terminal part of the protein. The original start codon and the leader sequence for the protein are missing.

To compensate for these missing regions, two different constructs were made and tested for expression. In the first construct, an in-frame start codon was generated by deleting part of the polylinker in the basic vector for recombination pUC8-TK-7.5K (Fig. 24c). The vector was digested with EcoRV and SmaI, deleting part of the polylinker (Fig. 24d), and then religated. By this manipulation, the ATG codon contained in the SphI restriction site was placed in the correct reading frame for the coding region of the oocyst protein on the EcoRI fragment. The successful manipulation was confirmed by sequencing the new polylinker fragment. The predicted N-terminal amino acid sequence of the protein encoded by this construct is shown in Fig. 25A.

To compensate in the DNA sequence not only for the start codon but also for the missing leader sequence, the EcoRI fragment was placed in the correct reading frame behind a leader sequence of a malarial antigen. The malarial antigen used to isolate the leader sequence was the 190 kd protein described as Ro-33 [Certa et al., EMBO J. 6:4137 (1987)]. It must be noted, however, that other leader sequences such as coccidial leader sequences could be used for the same purpose.

To isolate the DNA fragment containing the leader sequence, the Ro-33 DNA was digested with DraI. The fragment containing the recognition sites for the restriction enzymes PvuII and HindIII was isolated and digested with HindIII. The original vector construct pUC8-TK-7.5K (Fig. 24c) was cleaved with SalI, treated with Klenow and then digested with HindIII. In this vector fragment, the isolated DraI-HindIII malarial antigen leader fragment was cloned. This construct was used to express a fusion protein between the P. falciparum 190 kd protein and the E. tenella antigen recognized by antibody 8A2 and encoded by the EcoRI fragment. The predicted N-terminal amino acid sequence of this fusion protein is shown in Fig. 25B.

The EcoRI fragment encoding the 28 kd protein was cloned into the EcoRI site of the polylinker contained in the basic vector (Fig. 24c). Constructs containing the fragment in the correct orientation were propagated and used for recombination into the vaccinia virus.

Because of the way that the initiation site for translation of the fragment was engineered into the basic vector (see above), two different N-terminal sequences were expected for the gene to be expressed by the recombinant vaccinia virus (Fig. 25). While only 3 additional amino acids (Met, Arg, and Trp) are added to the original sequence in the first construct (Fig. 25A), in the second a total of 47 amino acids derived from the leader sequence of the 190 kd malarial antigen are fused to the N-terminus of the polypeptide recognized by monoclonal antibody 8A2 (Fig. 25B). By processing at the potential cleavage site of the leader sequence, 19 of the additional amino acids are removed, leading to a maturated protein starting with Val, Thr, His.

The EcoRI fragment coding for the 20 kd protein recognized by monoclonal antibody 6A5 contains the entire sequence. This fragment, without further manipulation, was cloned into the EcoRI site of the VV vector as described above.

Recombination of the above genes coding for coccidial antigens into a strain WR vaccinia virus was carried out using a two-step procedure for selection of the recombinant virus.

In the first step, CVI monkey cells were grown in medium I [Eagle's Minimal Essential Medium (MEM), 5% fetal calf serum (FCS; from Amimed), Penicillin/Streptomycin (100 units/ml and 100 µg/ml, respectively) and 2 mM glutamine; all reagents from Gibco] in 8 cm^2 culture plates to 80-90% confluency. The medium was removed and replaced with 0.2 ml of a virus suspension containing the temperature sensitive vaccinia virus strain ts N7 [Drillen et al., Virology 131:385 (1983)] at 0.1 plaque forming units (pfu)/cell. The plates were left at room temperature for 1 hour, after which 2 ml of Medium I were added to each plate and the plates were incubated for 2 hours at 33 °C (the growth permissive temperature for this virus) in a CO_2 incubator [Kieny et al., Nature 312:163 (1984)].

One half hour before the end of the above incubation period, a DNA-containing calcium-phosphate precipitate was prepared. This contained HeBS buffer [280 mM NaCl, 1.5 mM sodium-hydrogen-phosphate, 50 mM HEPES], 200 ng of purified vaccinia strain WR virus DNA and 200 ng of purified coccidial antigen-gene containing plasmid DNA, in a total volume of 0.55 ml. Each DNA was added in 1 µl of TE-buffer (10 mM Tris-HCl, pH 7. 5, 1 mM EDTA) To this solution was added, drop-wise and with gentle swirling, 0.55 ml of a 250 mM calcium-chloride solution. This mixture was left at room temperature for 20 minutes.

After the 2 hour incubation, the medium from the culture plates was aspirated and replaced with 0.25 ml of the above DNA-containing calcium precipitate and left at room temperature for one hour. Subsequently, 2 ml of Medium I was added to each plate, and the plates were incubated for 2 hours at 39.5 °C in a 5% CO_2 incubator (Kieny et al., supra). At this temperature, the ts N7 virus cannot replicate, resulting in a selection for viruses which have recombined at least in the ts7 locus. Because the calcium-phosphate is eventually inhibitory to cell growth, the medium was removed after the above 2 hour incubation, and the cells were washed 3 times with 1 ml of PBS under gentle swirling. The final PBS solution was aspirated, and 2 ml of Medium I were added to each plate. Incubation at 39.5 °C in a CO_2 incubator was continued for 2 days.

After the two-day incubation, the culture plates with the medium and cells were placed at -30 °C for a short time and then thawed, the still attached cells were scraped from the bottom of the plate and the suspension was sonicated as described above. This homogenate was used for the second selection step.

In this step, medium from a nearly confluent lawn of human 143B TK cells (ATCC CRL 8303) growing in 8 cm^2 culture plates was removed and replaced with 0.2 ml of undiluted homogenate or homogenate diluted 1:5 or 1:30 (vol/vol) with PBS. Infection of the TK^- cells was allowed to proceed at room temperature for 1 hour.

After the incubation, 2 ml of semi-solid Medium II (Medium I with non-essential amino acids (GIBCO; order number 043-1140), essential vitamins (GIBCO; order number 042-1120) and 1% agarose) containing 0.1 mg/ml bromodeoxyuridine (BUdR, Sigman Chemical Co. were added to the cells. The plates were then incubated for 16-24 hours at 37 °C in a CO_2 incubator. A second layer of semi-solid Medium II, containing 0.2% neutral red in addition to the above components, was placed over the cells and the plates were incubated for another 16-24 hours. Colorless plaques appeared which were clearly visible, and the virus was recovered as individual clones by piercing the plaque region with a Pasteur pipette (plaque purification). Virus recovered in this way was grown on CVI cells as described above and subjected to a second and third round of plaque purification on 143B TK^- cells. These plaque-purified viruses were grown and purified as described above.

To test for the expression of the coccidial antigen by the recombinant virus, CVI cells infected with recombinant virus were sedimented in a table-top centrifuge (Hettich Mikrorapid K, 100% for 3 minutes at 20 °C), and the pellet was washed twice with PBS, recentrifuged and resuspended in PBS. The cell

suspension was applied to a glass microscope slide (Flow) and allowed to dry. A second method consisted of growing the CVI cells directly on microscope slides (Miles Lab-Tek 4808), infecting the cells with virus and incubating for 1-2 days. The cells were then washed free of growth medium with PBS and allowed to dry on the slides at room temperature. To fix the cells, the slides were submerged in acetone for at least one hour at -30° C and allowed to dry at room temperature.

Mouse anti-coccidial antigen monoclonal antibodies diluted in PBS were layered onto the microscope slides so that the cells were evenly covered with liquid. The slides were placed in a humid chamber at 37° C for one hour and subsequently washed several times with PBS. Without allowing the slides to dry, a second antibody (FITC labeled goat anti-mouse IgG, Nordic) also diluted in PBS was layered onto the slides, and the slides were placed in a humid chamber at 37° C for one hour to allow the antibodies to react. After several washes with PBS, the slides were allowed to dry completely. A few drops of 20% (vol/vol) glycerine in water were pipetted onto the slide, and a cover glass (Menzel 24x60) was placed on top. The fluorescence of the cell preparation was then monitored under UV light in a microscope (Zeiss ICM 405, F10 or Planapo 63 objective).

The WR strain virus can multiply in almost all cell types [Drillen et al., J. Virology 28:843 (1978)], and its multiplication can be observed directly through the purification on 143B TK$^-$ cells. These plaque-purified viruses were grown and purified as described above.

To test for the expression of the coccidial antigen by the recombinant virus, CV1 cells infected with recombinant virus were sedimented in a table-top centrifuge (Hettich Mikrorapid K, 100% for 3 minutes at 20° C), and the pellet was washed twice with PBS, recentrifuged and resuspended in PBS. The cell suspension was applied to a glass microscope slide (Flow) and allowed to dry. A second method consisted of growing the CV1 cells directly on microscope slides (Miles Lab-Tek 4808), infecting the cells with virus and incubating for 1-2 days. The cells were then washed free of growth medium with PBS and allowed to dry on the slides at room temperature. To fix the cells, the slides were submerged in acetone for at least one hour at -30° C and allowed to dry at room temperature.

Mouse anti-coccidial antigen monoclonal antibodies diluted in PBS were layered onto the microscope slides so that the cells were evenly covered with liquid. The slides were placed in a humid chamber at 37° C for one hour and subsequently washed several times with PBS. Without allowing the slides to dry, a second antibody (FITC labeled goat anti-mouse IgG, Nordic) also diluted in PBS was layered onto the slides, and the slides were placed in a humid chamber at 37° C for one hour to allow the antibodies to react. After several washes with PBS, the slides were allowed to dry completely. A few drops of 20% (vol/vol) glycerine in water were pipetted onto the slide, and a cover glass (Menzel 24x60) was placed on top. The fluorescence of the cell preparation was then monitored under UV light in a microscope (Zeiss ICM 405, F10 or Planapo 63 objective).

The WR strain virus can multiply in almost all cell types [Drillen et al., J. Virology 28:843 (1978)], and its multiplication can be observed directly through the formation of plaques. In most cases, however, chicken embryo fibroblast (CEF) cells were used to prepare large stocks of the virus.

To obtain CEF cells, 11-day old embryos were isolated from eggs, freed from their extremities, cut into small pieces and resuspended in a 0.25% trypsin solution (Difco) for 2 hours at room temperature. This suspension was diluted with one volume of Medium I and filtered through a cell sieve (Bellco, 150 mesh), and the cells were sedimented (Hermie table-top centrifuge, 5 minutes, 2,000 rpm, room temperature). The cell pellet was resuspended in 1/4 of the original volume of Medium I and this CEF cell suspension inoculated into cell culture plates. Depending on the starting cell density, the cultures were allowed to grow 1-2 days and used for infection directly or after 1-2 further passages. A synopsis for the establishment of such primary cultures can be found in Frehney, Culture of Animal Cells, Alan R. Liss Verlag, New York 1983, Chapter 11, p. 99.

For infection, the medium was removed from 80-90% confluent CEF cells growing in 175 cm culture flasks (Falcon 3028), and the cells were incubated in a PBS solution containing virus (0.1 pfu/cell, 0.01 ml/cm^2) for one hour at room temperature (20° C) (PBS/Dulbecco, Amimed). Medium I was then added (0.2 ml/cm^2), and the flasks were incubated at 37° C for 2-3 days until about 80% of the cells had lysed. The resulting stock solution was stored directly with cells and medium in the original culture flasks at -30° C before virus purification.

The following purification steps were used to obtain a virus preparation free of all host cell specific components. Infected cell cultures which had been stored at -30° C were thawed and the remaining cells were freed from the surface of the flask by shaking or scraping. The cells and virus were centrifuged out of the medium (Sorvall centrifuge, GSA rotor, 1 hour at 5,000 rpm, 10° C). The pellet of cells and virus particles was resuspended in PBS (10-20 X the volume of the pellet) and recentrifuged as above. This pellet was then resuspended in a 10-fold volume of RSB buffer (10 mM Tris-HCl, pH 8.0, 10 mM KCl, 1 mM

$MgCl_2$).

To lyse the remaining intact cells and free the virus from the cell membranes, the above suspension was subjected to sonification (twice, 10 seconds at 60 watts, room temperature, Labsonic 1510 with 4 mm probe). The mixture was then centrifuged in a Sorval GSA rotor for 3 minutes at 3,000 rpm, 10° C. A virus suspension free from cell nuclei and large cell debris was thus produced. The supernatant was carefully removed, and the pellet was resuspended in RSB buffer, resonicated and centrifuged as above.

The second supernatant was combined with the first, layered onto a 10 ml 35% sucrose cushion (Fluka, in 10 mM Tirs-HCl, pH 8.0) and centrifuged for 90 minutes at 14,000 rpm in a Kontron TST 28.38/17 rotor (Beckman SW 27 analog) at 10° C. The supernatant was decanted and the pellet of virus particles was resuspended in 10 ml of 10 mM Tris-HCl, pH 8.0, sonicated to homogenize the mixture (2 times for 10 seconds at room temperature as described above) and loaded onto a step gradient for further purification.

The step gradient consisted of 5 ml aliquots of sucrose in 10 mM Tris-HCl, pH 8.0, of the following concentrations: 20%, 25%, 30%, 35% and 40%. This gradient was centrifuged in a Kontron TST 28.38/17 rotor for 35 minutes at 14,000 rpm, 10° C. Several bands containing virus particles were visible in the 30%-40% sucrose region. This region of the gradient was removed (10 ml), the sucrose solution was diluted with PBS (20 ml) and the virus particles were sedimented (Kontron rotor, 90 minutes at 14,000 rpm, 10° C). The pellet contained almost exclusively virus particles (as judged by comparison of OD measurement and plaque assay, see below). This pellet was resuspended in PBS so that the virus concentration was on the average 1-5 x 10^9 pfu/ml. This virus stock was used either directly or diluted with PBS.

To determine the virus concentration and the purity of the virus stock, two methods were used. The absolute concentration of virus particles was conveniently obtained by measuring the optical density (OD) of the stock solution in a spectrophotometer (Uvikon 860) at 260 nm (OD/260), where 1 OD/260 equals about 1.2 x 10^{10} particles per ml [Joklik, Virology 18:9 (1962)]. Virus concentration was also obtained by titrating the virus on cells (plaque assay), assuming that only one out of 60 virus particles can infect a cell.

To titer the virus concentration on cultured cells, CEF cells were grown in Medium I on 8 cm^2 plastic culture plates (Falcon 3001). When the cells had reached 80-90% confluency, the medium was removed, replaced with 0.2 ml of a diluted virus solution in PBS, and left at room temperature for 1 hour. The virus stock solution was diluted in 10-fold steps. After the room temperature incubation, 2 ml of semi-solid Medium I (Medium I +- 1% agarose) were added to each plate, and the plates were placed for 16-24 hours in a CO_2 incubator. Subsequently, 2 ml of semi-solid Medium I containing 0.2% neutral red (Fluka 72210) was layered on to stain the living cells, and the plates were incubated for an additional 16-24 hours. The colorless plaques were then counted under a microscope.

7.2.2. CHICK IMMUNIZATION

To determine whether vaccinia viral vectors harboring genes coding for the E. tenella proteins which specifically bound to monoclonal antibodies 8A2 and 6A5 could protect chicks against challenge by sporulated oocysts of a pathogenic strain of E. tenella (strains T2-750/7, T7-776/1 or T6-771), the following tests were carried out.

All tests were conducted using cockerels of a layer breed (Warren) supplied by the hatchery E. Wuthrich, Belp, Switzerland. Day-old chicks were reared in heated battery-brooders until the indicated ages, after which they were divided into various test groups and maintained coccidiosis-free in wire-floored cages. Throughout the tests, a commercial broiler-grower diet, based on maize, wheat and soybean meal (crude protein 21.7%) was fed.

In the first test, on day 42 chicks of equivalent weight were randomly divided into three groups of six birds each. Three days later, the chicks were immunized with either recombinant or wild type Vaccinia virus by means of two injections of 50 µl each of the respective virus suspension (10^{10} pfu/ml in PBS) given subcutaneously into the right wing web. Two recombinant vaccinia viruses were used, both of which contained DNA coding for the E. tenella protein which specifically bound to antibody 8A2. One of the viruses (designated 37K M3) contained the leader sequence of the 190 kd malarial antigen; the other virus (designated 37K K3) lacked this leader sequence. The wild type vaccinia strain WR served as a negative control.

One week after the first injection, a booster injection was made into the left wing web of the chicks under identical conditions, using the same type of vaccinia virus used previously. One week after the boost (day 59), blood samples of 2 ml each were taken from all the chicks and the serum was assayed for the presence of specific antibodies by ELISA. Briefly, the wells of a microtiterplate (NUNC Immunoplate F96)

were coated with a suspension of sporozoites of E. tenella (10'000 cells/m1) and incubated with the chicken serum at increasing dilution rates. As detecting agents goat anti-chicken immunoglobulins conjugated to horseradish-peroxydase (Kirkegaard and Perry Laboratory, Gaithersburg, U.S.A.) were used together with tetramethylbenzidine as substrate. The developing blue color was read in a Titertek Multiskan MCC/340 MkII at a wavelength of 450 nm. The titer was defined as the reciprocal value of that serum dilution, giving an optical density of at least double the background value (table 7).

Four weeks after the first injection (day 73) the chicks were challenged with 50,000 of the sporulated oocysts. The inoculum of coccidial, which was suspended in 1 ml of physiological saline, was administered orally into the crop of the chicks by means of a blunt needle on a calibrated syringe. On day 80, all of the chicks were sacrificed, necropsied and scored for gross lesions in the ceca (score 0 = normal, 1 = slight infestation, 2 = medium infestation, 3 = severe lesions, 4 = chick died from coccidiosis). The droppings were collected quantitatively over the last two days of the infectious cycle, and the number of excreted oocysts was determined in a representative sample of feces. The results are shown in Table 7.

TABLE 7

VACCINATION OF 45 DAY OLD CHICKS WITH VACCINIA VIRUSES EXPRESSING THE 28 KD PROTEIN				
Virus[a]	Number of Chicks	Antibody Titer	Cecal Lesion Score	Daily Oocyst Excretion/Chick ($x10^{-6}$)
37 K3	6	430	1.33	149
37 M3	6[b]	1360	1.50	107
Wild-Type	6	200	2.67	271

a- Virus 37 M3 contained the leader sequence of the 190 kd malarial antigen; virus 37 K3 did not. The wild-type virus was vaccinia strain WR.

b- Two chicks were sacrified prior to coccidiosis challenge.

Table 7 shows that compared to the wild-type control virus, both viruses containing DNA coding for the 28 kd protein induced the production of antibodies specific to the parasite and conferred some protection against oocyst challenge, both in terms of a reduction of cecal lesion score and reduced oocyst excretion. Regarding protection against coccidiosis the two viruses were equally effective, however the construction with the malarial leader (37 M3) generated a higher antibody titer against the sporozoite antigen then the standard virus construction (37 K3) in the chick, indicating an advantage for the fusion construct.

In the second test, cockerels were reared and immunized as described above but beginning at day 22. A viral dose of 2×10^8 pfu in 100 ml of PBS was administered at that time. The viruses used were from different preparations of vaccinia virus containing DNA coding for the 28 kd protein without (designated 37 K5) or with (designated 37 M19) the malarial leader sequence. The same wild-type strain WR virus was used as a control. Booster injections into the right wing webs at the same doses were given either one or 2 weeks after the first injections.

On day 57 (5 weeks after the first injection), all of the chicks were challenged with 50,000 of the sporulated oocysts. One week later, the chicks were sacrificed, necropsied and scored as described above. Daily weight gain following infection and oocyst excretion during the final two days were also determined. The results are shown in Table 8.

TABLE 8

VACCINATION OF 22 DAY OLD CHICKS WITH VACCINIA VIRUSES EXPRESSING THE 28 KD PROTEIN					
Virus[a]	Time to Booster (Weeks)	Number of Chicks	Daily Weight Gain (g)	Cecal Lesion Score	Daily Oocyst Excretion/Chick (x 10^{-6})
37 K5	1	8	11.45	2.38	21.0
37 M19	1	8	15.61	2.13	24.0
Wild-Type	1	8	8.77	2.50	33.1
37 K5	2	8	5.14	2.25	22.3
37 M19	2	8	11.79	2.13	32.7
Wild-Type	2	8	8.34	2.63	37.0

a- Virus 37 M19 contained the leader sequence of the 190 kd malarial antigen. Virus 37 K5 did not. The wild-type virus was vaccinia strain WR.

Table 8 shows that, compared to the wild-type control virus, both viruses containing the coccidial DNA conferred some protection against the pathogenic oocyst challenge, in terms of weight gain, cecal lesion score and oocyst excretion. Both viruses were about equally effective. Administration of the booster 1 week after the primary injection produced somewhat better weight gain and lower oocyst excretion, but cecal lesion scores were about the same for both booster schedules.

In the third test, the effect of three vaccinations was examined. Chicks were injected (right wing web) with two 50 µl aliquots (3 x 10^9 pfu/ml) of suspensions of wild-type vaccinia virus or virus containing DNA coding for the 20 kd protein which bound specifically to monoclonal antibody 6A5, at 21 days of age. All chicks were given same dose booster injections at day 28 into the left wing webs. Some chicks were given additional same-dose booster injections into the wing webs of both sides at day 35. Other chicks were maintained with no vaccinations, as further controls.

On day 42, blood samples were taken from all the chicks and assayed by ELISA for the presence of specific antibodies against the sporozoite stage of the parasite as previously described. On day 49 (4 weeks after the first injections), all of the chicks were challenged with 50,000 of the sporulated oocysts. One week later, the chicks were sacrificed, necropsied and scored for gross cecal lesions as described above. Body weight was recorded weekly for calculation of daily weight gain, and the droppings were collected over the last two days of the infectious cycle to determine oocyst excretion. The results are shown in Table 9.

TABLE 9

VACCINATION OF 21 DAY OLD CHICKS WITH VACCINIA VIRUS EXPRESSING THE 20 KD PROTEIN						
VV	No. of Injections	Number of Chicks	Antibody Titer	Daily Weight Gain (g)	Cecal Lesion Score	Oocyst Excretion/g Feces (x10^{-6})
rVV	2	6	210	2.9	2.33	1.69
rVV	3	6	7200	4.2	1.67	1.32
WT	3	6	560	-4.1	2.67	2.09
N	-	6	0	-3.8	2.83	1.75
rVV With Coccidial DNA WT Wild-Type N None						

The data of table 9 show that when injected three times the virus producing the coccidial antigen generated a high titer of antibodies specific against sporozoite proteins. Moreover, both treatments with this type of recombinant virus provided some protection against oocyst infection in terms of enhanced weight gain, reduced cecal lesion score and lowered oocyst excretion. Comparison of the results obtained with

unvaccinated controls shows that 0accination with the wild-type vaccinia virus did not confer protection. Therefore, the protection conferred by the virus harboring the coccidial DNA was specific and not due to a generalized immune stimulation caused by exposure to the vaccinia virus itself. Three vaccinations were more effective than two.

Many modifications and variations of this invention may be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

Claims

1. A protein having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen, which surface antigen has an apparent molecular weight of about 28, 37, 120 or greater than 200 kilodaltons and specifically binds to one or more monoclonal antibodies deposited with the American Type Culture Collection and assigned accession Nos. HB 9707 through HB 9712.

2. The protein of claim 1 which has the amino acid sequence shown in Fig. 15 or is a functional equivalent thereof.

3. The protein of claim 1 which has the amino acid sequence shown in Fig. 17 or is a functional equivalent thereof.

4. The protein of claim 1 which has the amino acid sequence shown in Fig. 19 or is a functional equivalent thereof.

5. The protein of claim 1 which has the amino acid sequence shown in Fig. 21 or is a functional equivalent thereof.

6. A DNA sequence encoding a protein according to any one of claims 1 to 5.

7. The DNA sequence of claim 6 comprising all or part of the nucleotide sequence shown in Fig. 14.

8. The DNA sequence of claim 6 comprising all or part of the nucleotide sequence shown in Fig. 16.

9. The DNA sequence of claim 6 comprising all or part of the nucleotide sequence shown in Fig. 18.

10. The DNA sequence of claim 6 comprising all or part of the nucleotide sequence shown in Fig. 20.

11. A recombinant vector comprising a DNA sequence according to any one of claims 6 to 10.

12. A recombinant vector according to claim 11, which recombinant vector is capable of directing expression of the DNA sequence in a compatible host organism.

13. The recombinant vector of claim 11 or 12 which is a poxvirus vector.

14. The recombinant vector of claim 11 or 12 which is an E.coli vector.

15. The recombinant vector of claim 14 which is pEV/2-4.

16. A host organism transformed with a recombinant vector according to any one of claims 11 to 15.

17. A transformed host organism according to claim 16, which transformed host organism is capable of expressing the DNA sequence encoding a protein as defined in any one of claims 1 to 5 comprised in said recombinant vector.

18. An antibody which is directed against a protein as claimed in any one of claims 1 to 5.

19. A antibody according to claim 18 which is a monoclonal antibody.

20. A monoclonal antibody according to claim 19 selected from the group consisting of ATCC Nos. HB 9707, HB 9708, HB 9709, HB 9710, HB 9711 and HB 9712.

21. A protein according to any one of claims 1 to 5 for the immunization of poultry against coccidiosis.

22. A process for the preparation of a protein according to any one of claims 1 to 5, which process comprises:

(a) culturing a host organism transformed with a recombinant vector comprising a DNA sequence encoding said protein under conditions in which the DNA sequence is expressed; and

(b) isolating the protein from the culture.

23. A process for the preparation of a transformed host organism according to claim 16 or 17, which process comprises transforming a host organism with a recombinant vector according to any one of claims 11 to 15 using methods known per se.

24. A vaccine for protecting poultry against coccidiosis comprising one or more proteins as defined in any one of claims 1 to 5 and a physiologically acceptable carrier.

25. A vaccine for protecting poultry against coccidiosis comprising a recombinant poxvirus vector according to claim 13.

26. The use of a protein according to any one of claims 1 to 5 for the preparation of a vaccine capable of protecting poultry against coccidiosis.

Claims for the following Contracting States : ES, GR.

1. A process for the preparation of a protein having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen, which process comprises:

(a) culturing a transformed host organism containing a recombinant vector comprising a DNA sequence encoding a protein having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen, which surface antigen has an apparent molecular weight of about 28, 37, 120 or greater than 200 kilodaltons and specifically binds to one or more monoclonal antibodies deposited with the American Type Culture Collection and assigned accession Nos. HB 9707 through HB 9712, under conditions in which the DNA sequence or fragment is expressed; and

(b) isolating the protein from the culture.

2. A process according to claim 1 wherein a transformed host organism containing a recombinant vector comprising a DNA sequence encoding a protein having the amino acid sequence shown in Fig. 15 or a functional equivalent thereof is used.

3. A process according to claim 1 wherein a transformed host organism containing a recombinant vector comprising a DNA sequence encoding a protein having the amino acid sequence shown in Fig. 17 or a functional equivalent thereof is used.

4. A process according to claim 1 wherein a transformed host organism containing a recombinant vector comprising a DNA sequence encoding a protein having the amino acid sequence shown in Fig. 19 or a functional equivalent thereof is used.

5. A process according to claim 1 wherein a transformed host organism containing a recombinant vector comprising a DNA sequence encoding a protein having the amino acid sequence shown in Fig. 21 or a functional equivalent thereof is used.

6. A process for the preparation of a transformed host organism capable of expressing a DNA sequence encoding a protein as defined in any one of claims 1 to 5, which process comprises transforming a host organism with a recombinant vector comprising said DNA in a manner known per se.

7. A process for the preparation of antibodies directed against a protein as defined in any one of claims to 5, which process comprises injecting said protein in a manner known per se into an animal which is capable of eliciting an immune response against said protein and isolating the antibodies produced by methods known in the art.

8. A process for the preparation of a vaccine for the immunization of poultry against coccidiosis, which process comprises mixing a protein as defined in any one of claims 1 to 5 with a pharmaceutically acceptable carrier.

9. A vaccine for the immunization of poultry against coccidiosis comprising one or more proteins as defined in any one of claims 1 to 5 and a physiologically acceptable carrier.

10. The use of a protein as defined in any one of claims 1 to 5 for the preparation of a vaccine capable of protecting poultry against coccidiosis.

11. A protein having one or more immunoreactive and/or antigenic determinants of an Eimeria surface antigen, whenever prepared by a process as claimed in any one of claims 1 to 5.

12. A transformed host organism capable of expressing a DNA sequence encoding a protein as defined in any one of claims 1 to 5, whenever prepared by a process as claimed in claim 6.

13. An antibody directed against a protein as defined in any one of claims 1 to 5, whenever prepared by a process as claimed in claim 7.

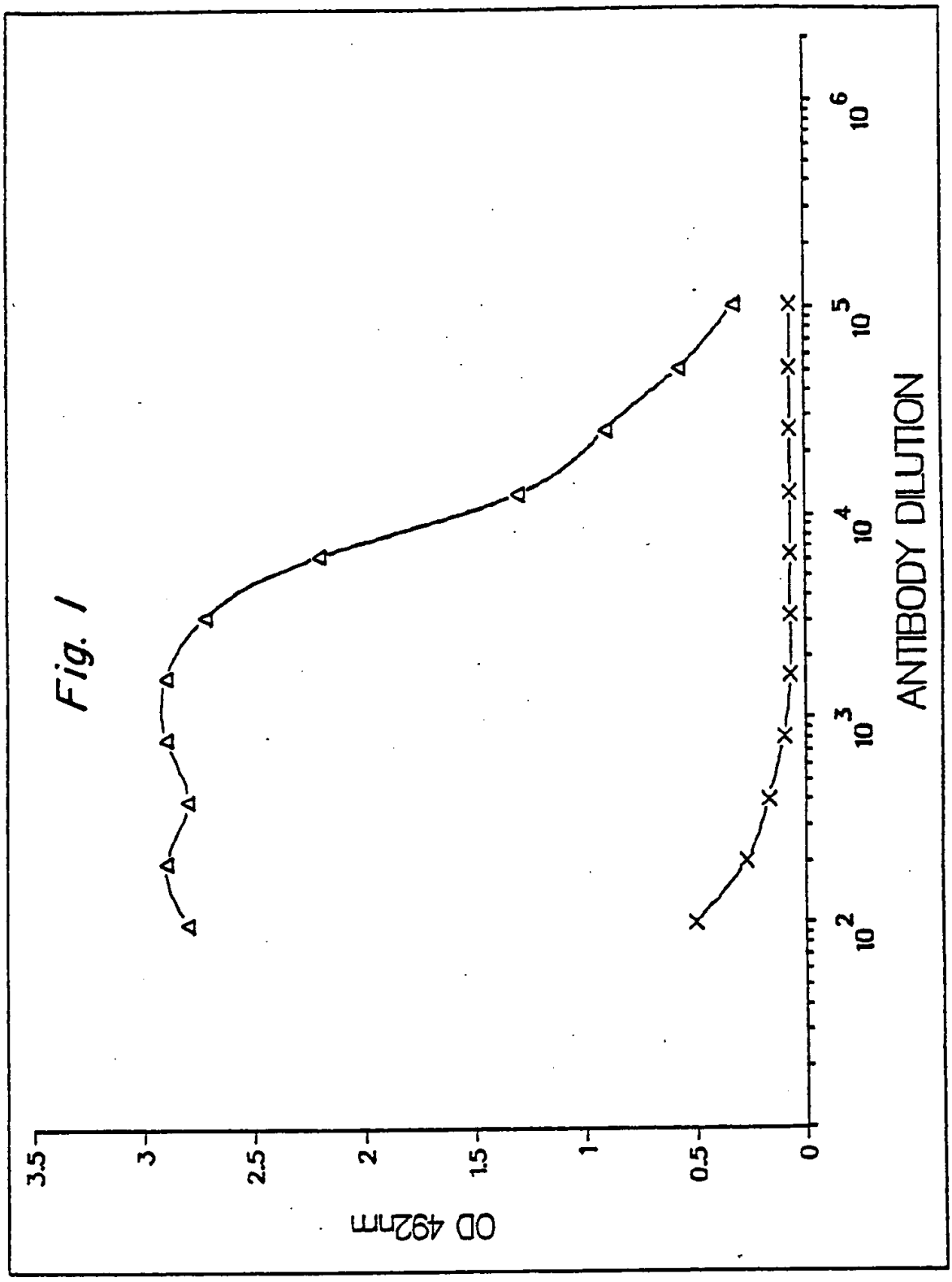

OD 492nm
3.5
3
2.5
2
1.5
1
0.5
0
10^2
10^3
10^4
10^5
10^6
ANTIBODY DILUTION

Fig. 1

Fig. 2

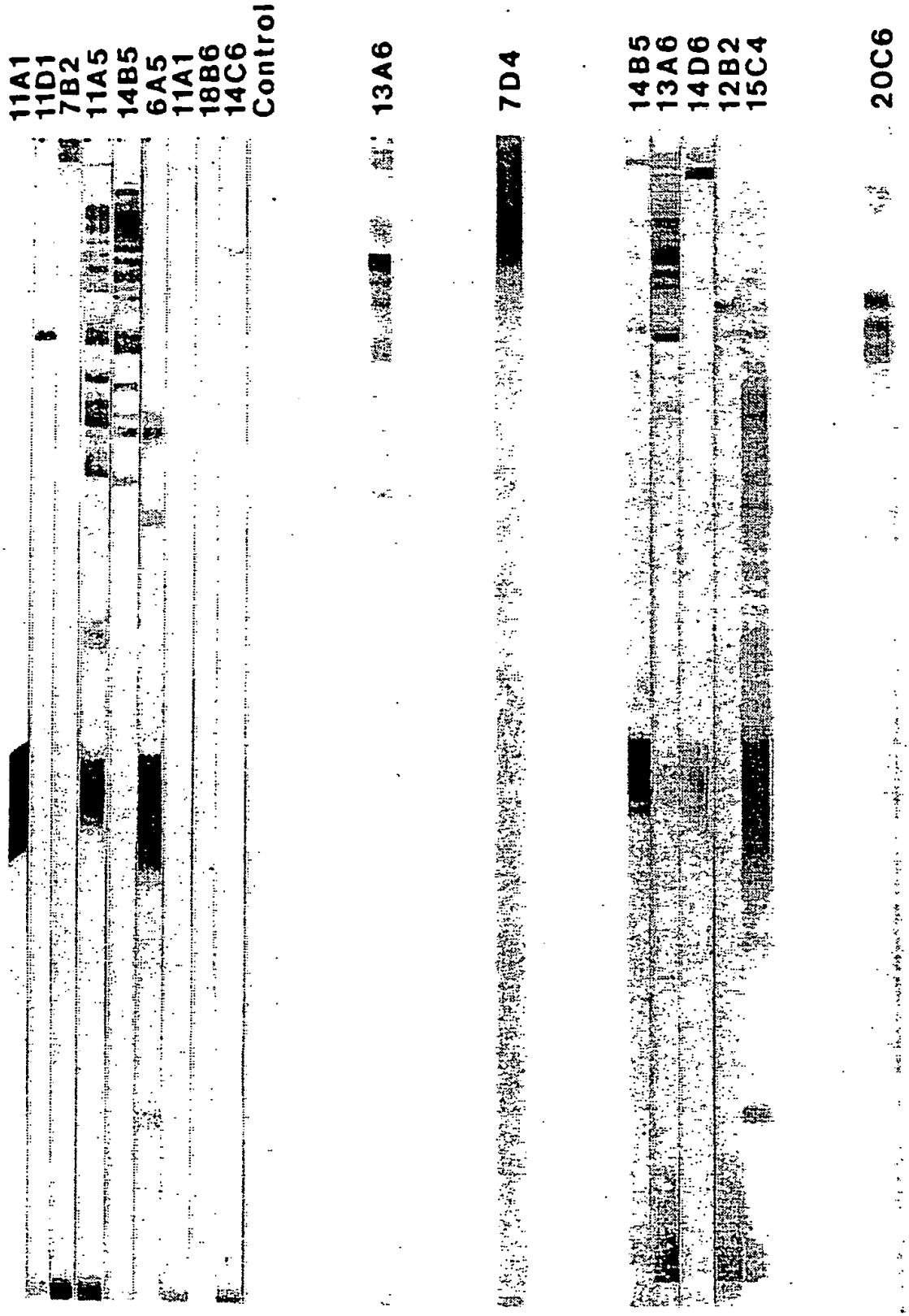

11A1
11D1
7B2
11A5
14B5
6A5
11A1
18B6
14C6
Control
13A6
7D4
14B5
13A6
14D6
12B2
15C4
20C6

Fig. 3

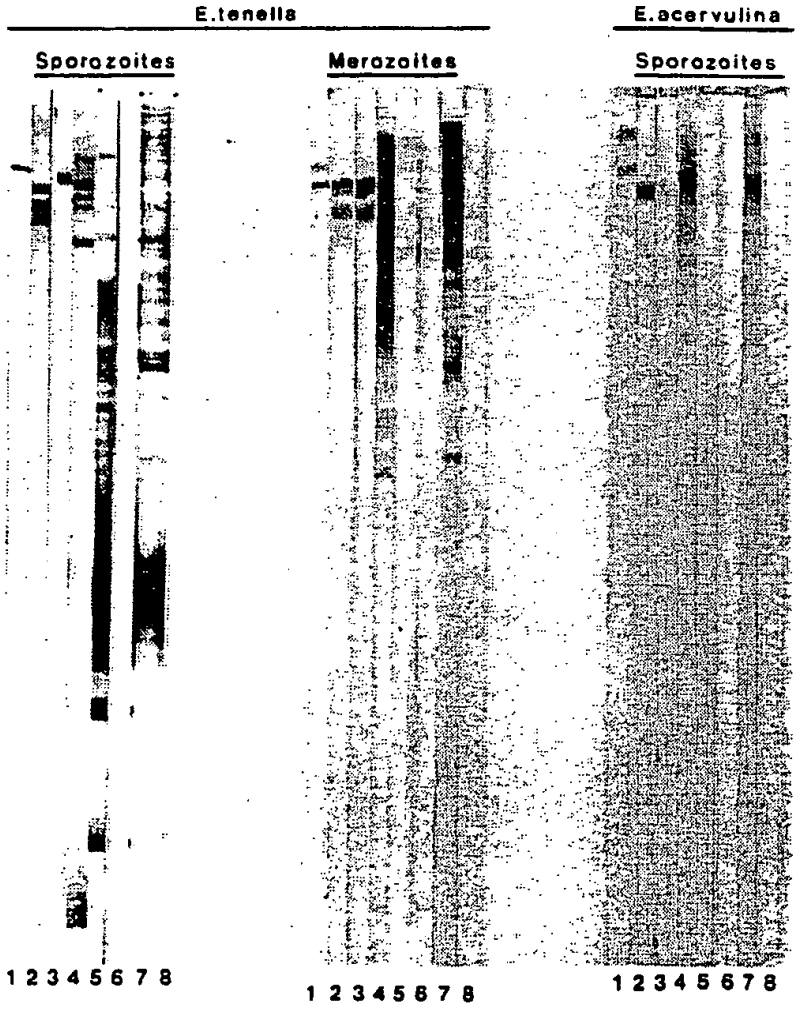
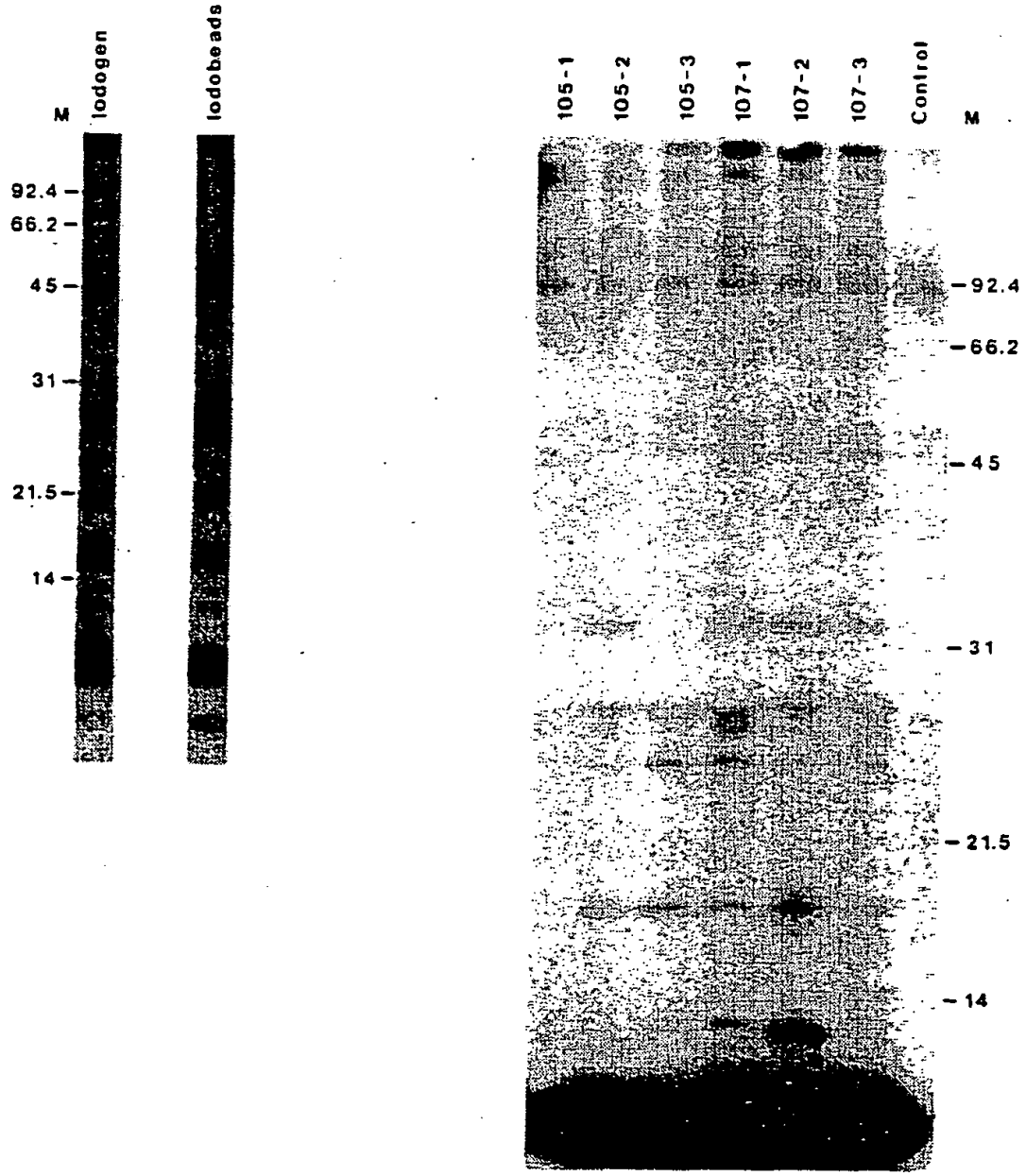

E.tenella
E.acervulina
Sporozoites
Merozoites
Sporozoites
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8
1 2 3 4 5 6 7 8

Fig. 4


M
Iodogen
Iodobeads
92.4
66.2
45
31
21.5
14
105-1
105-2
105-3
107-1
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107-3
Control
M
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31
21.5
14

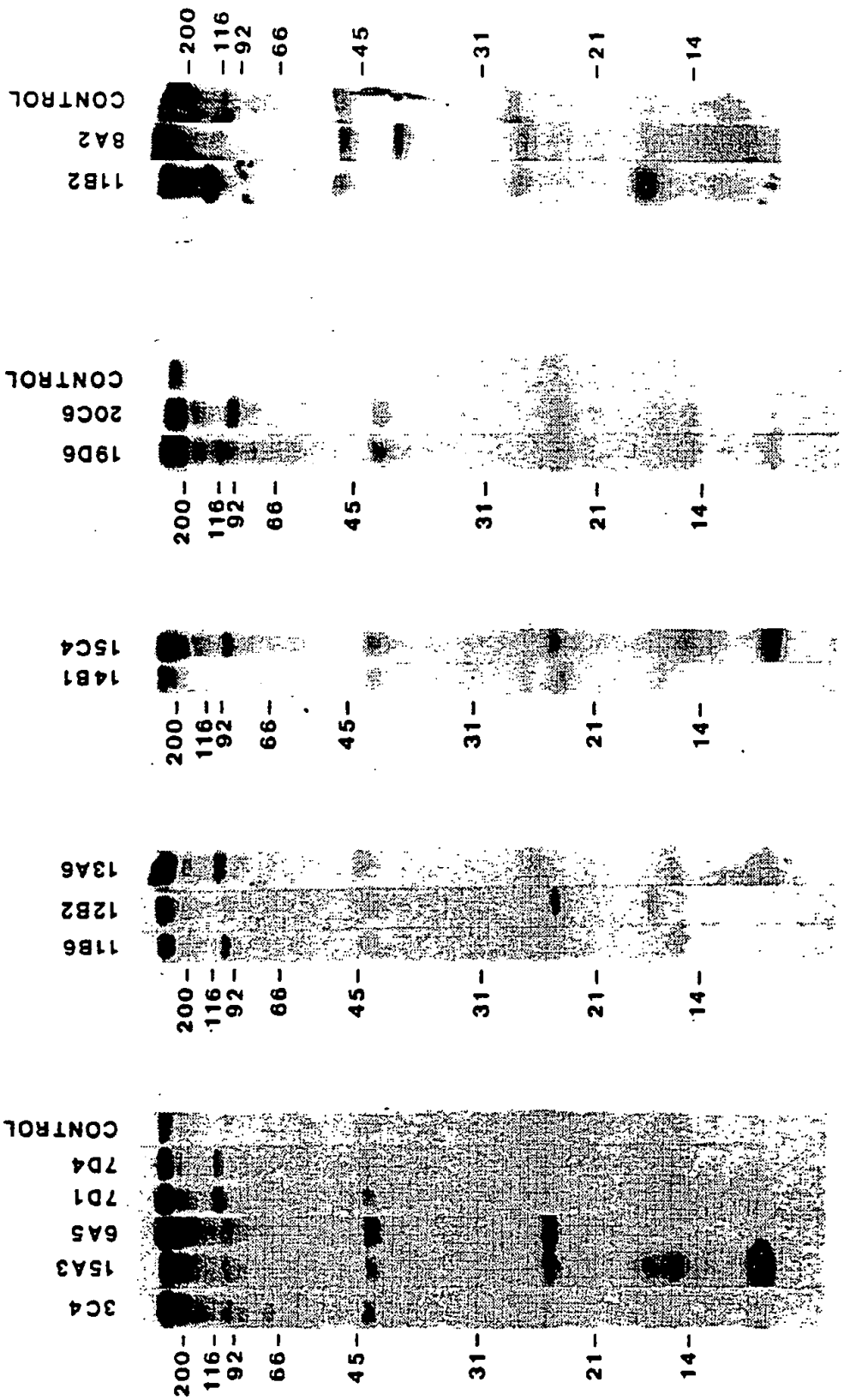

CONTROL
8A2
11B2
200
116
92
66
45
31
21
14
CONTROL
20C6
19D6
200
116
92
66
45
31
21
14
15C4
14B1
200
116
92
66
45
31
21
14
13A6
12B2
11B6
200
116
92
66
45
31
21
14
CONTROL
7D4
7D1
6A5
15A3
3C4
200
116
92
66
45
31
21
14

Fig. 5

Fig. 6

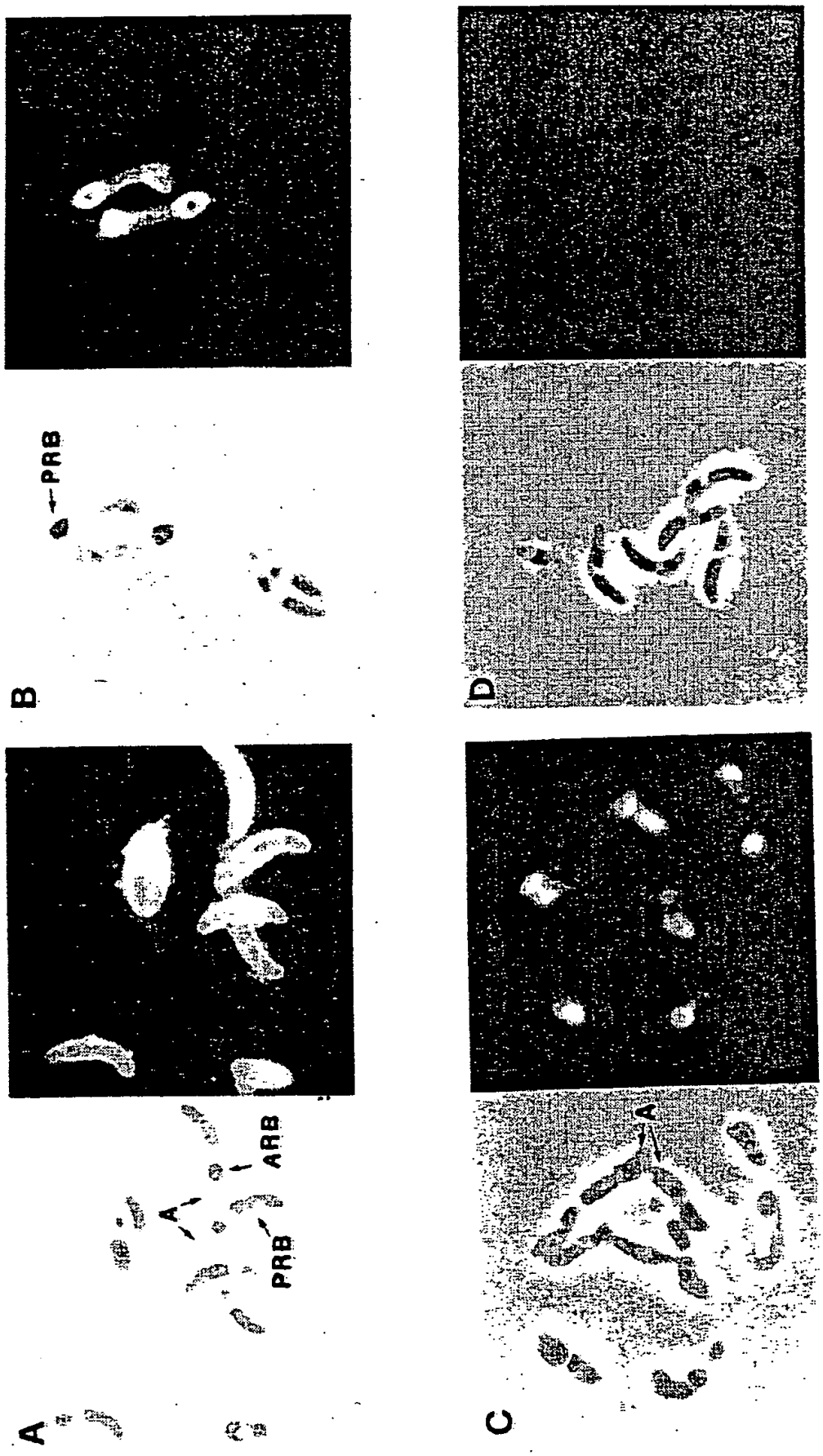
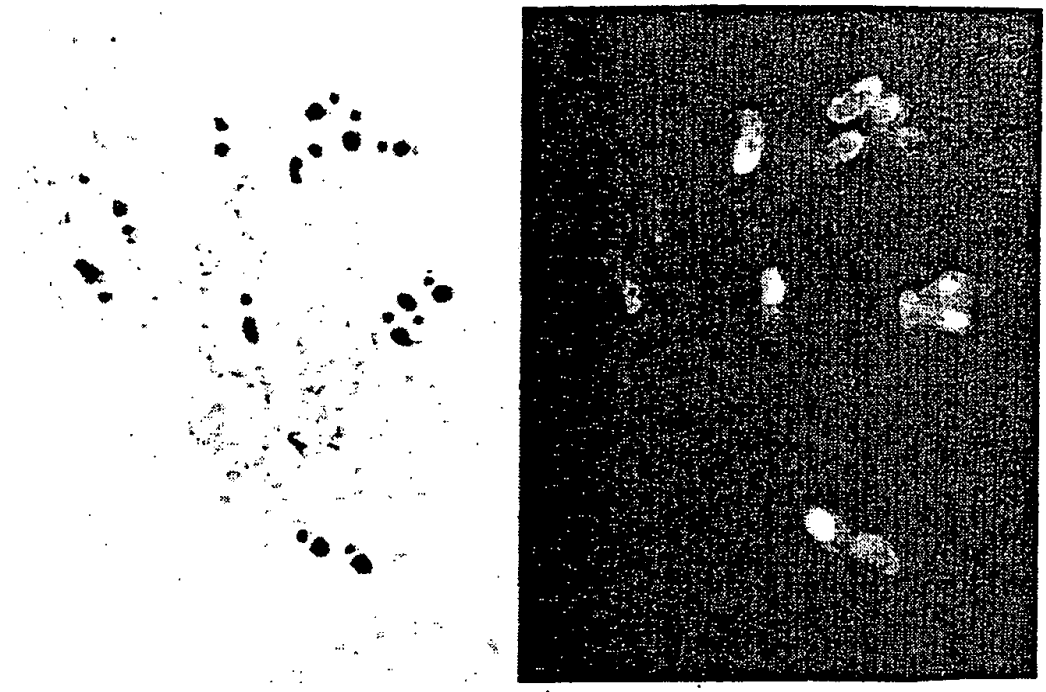
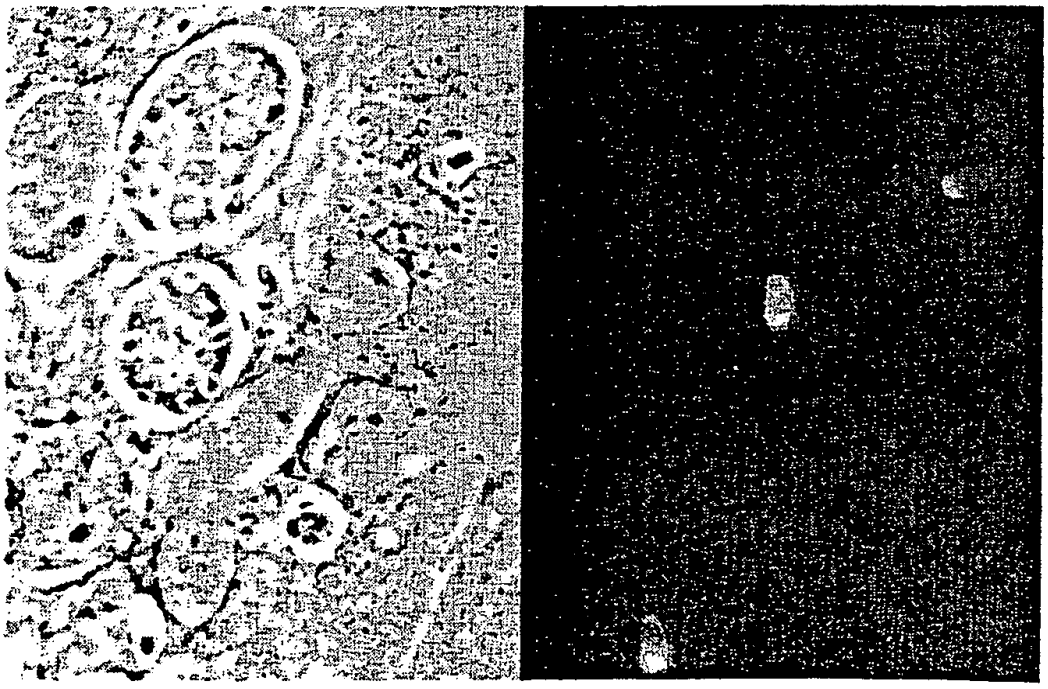


Fig. 7A

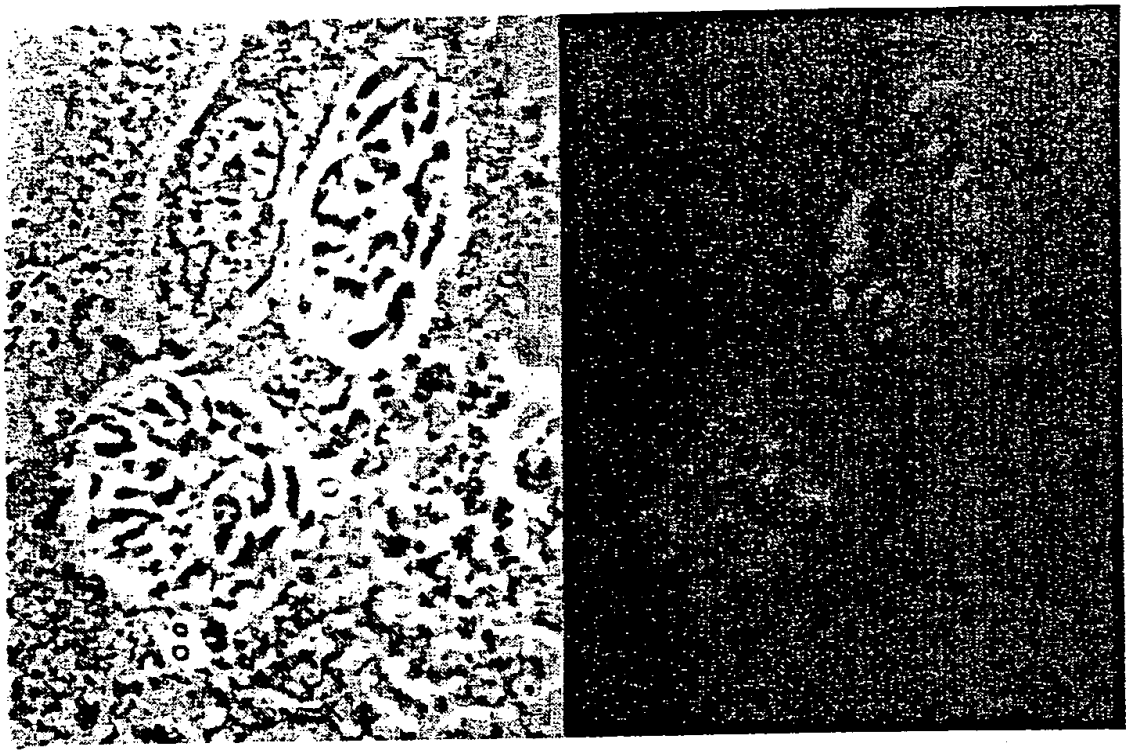


7D4 3 hrs



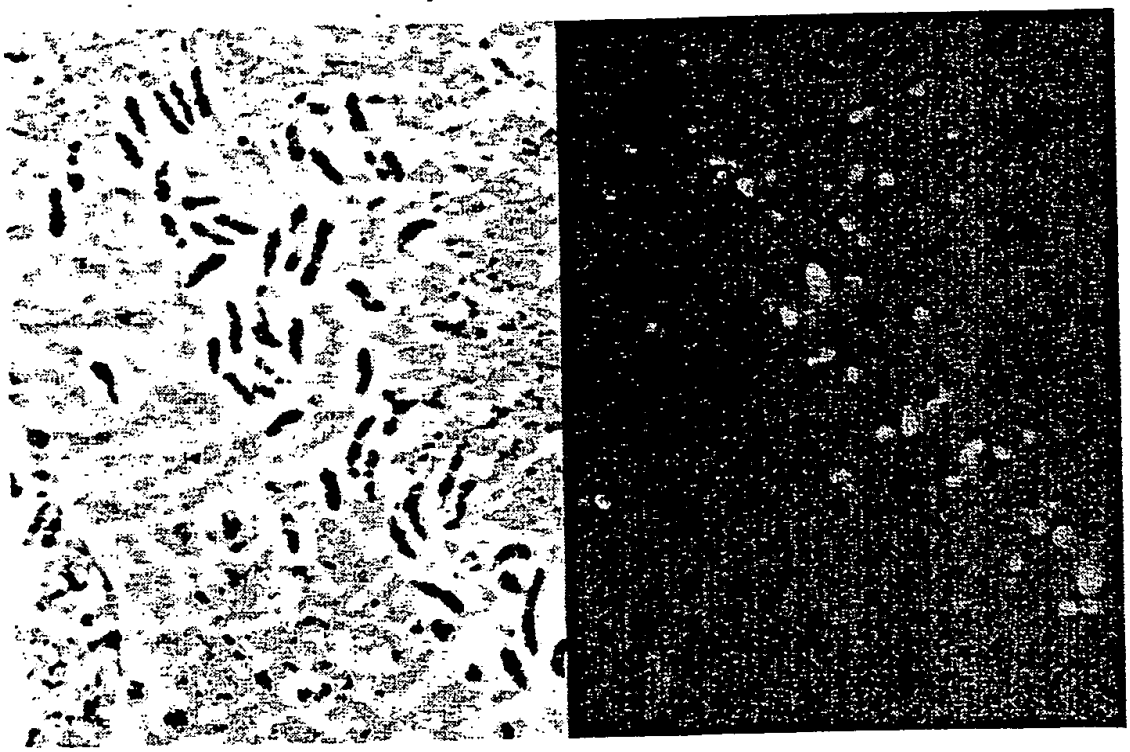
7D4 60 hrs

Fig. 7B



7D4

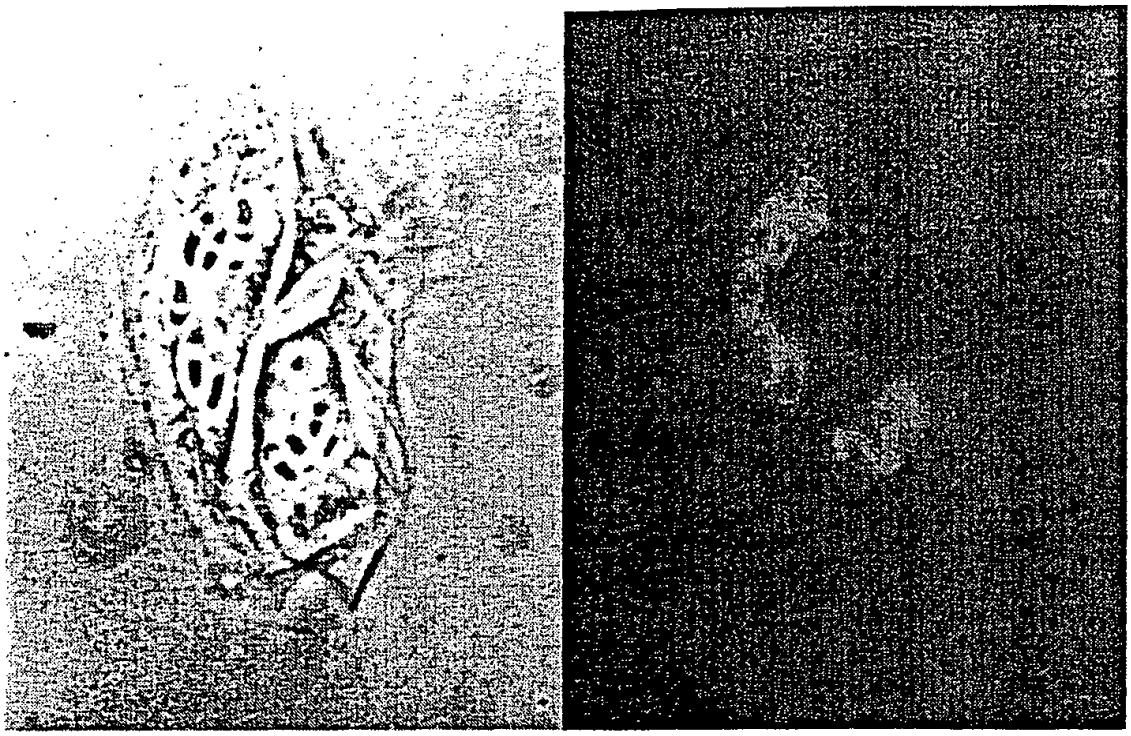
100 hrs



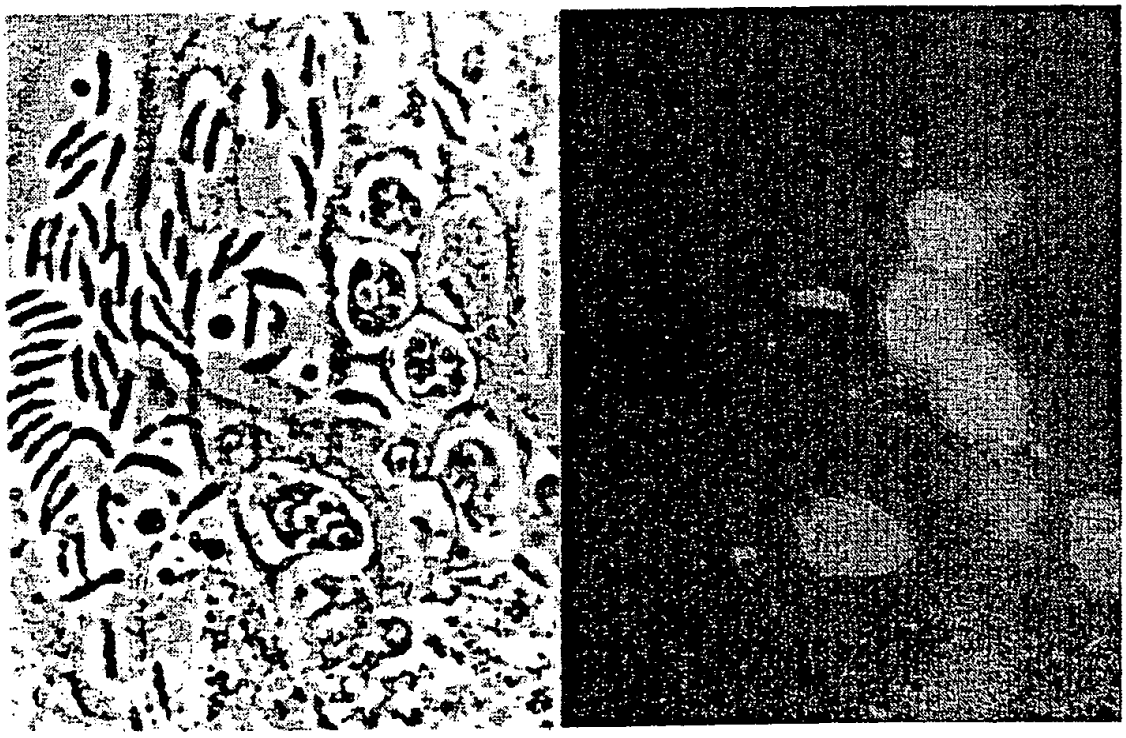
7D4

120 hrs

Fig. 7C

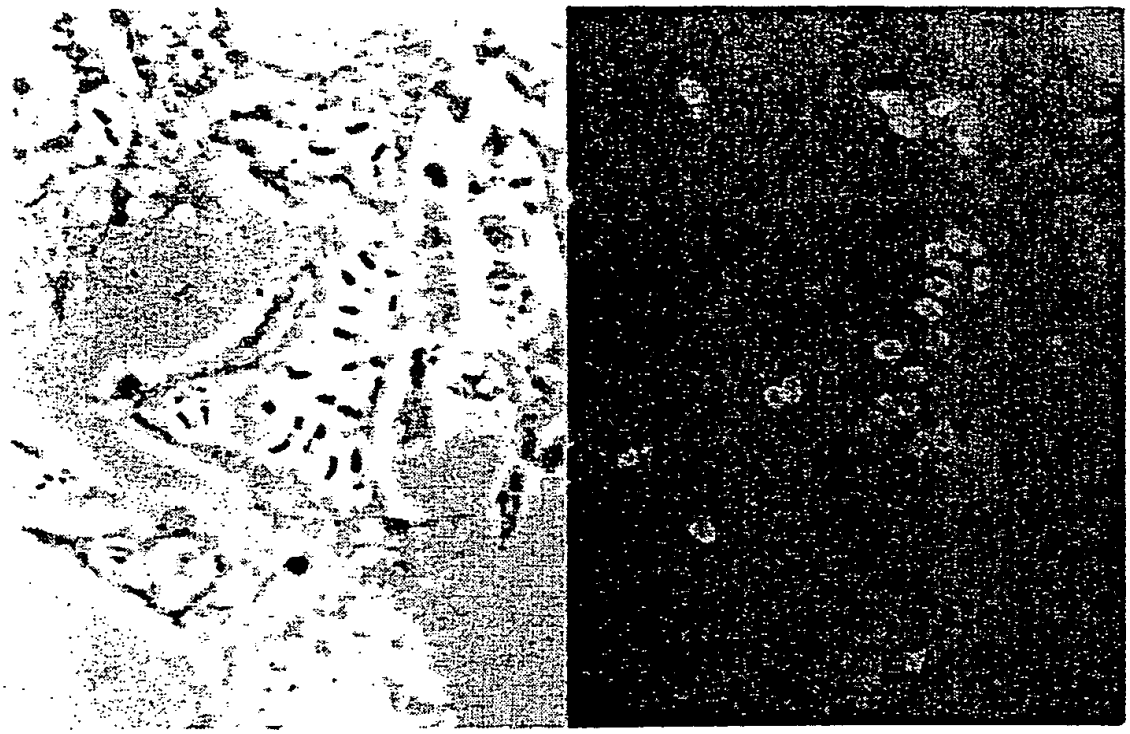


8A2 19 hrs

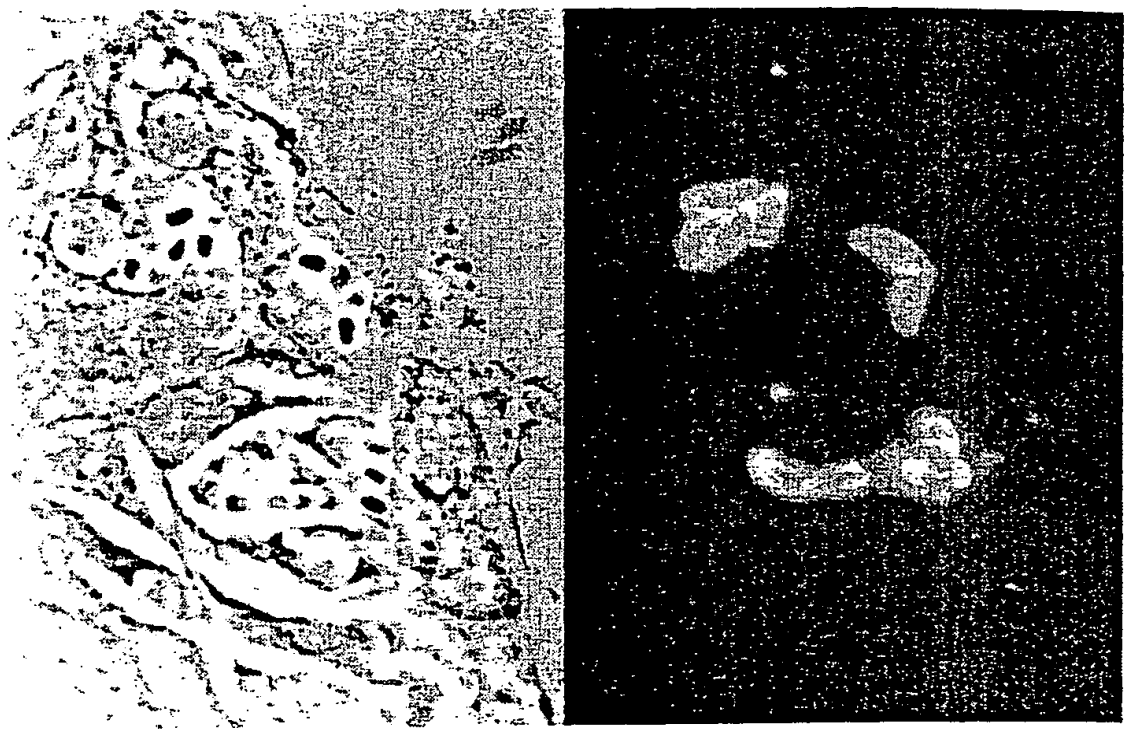


8A2 120 hrs

Fig. 7D



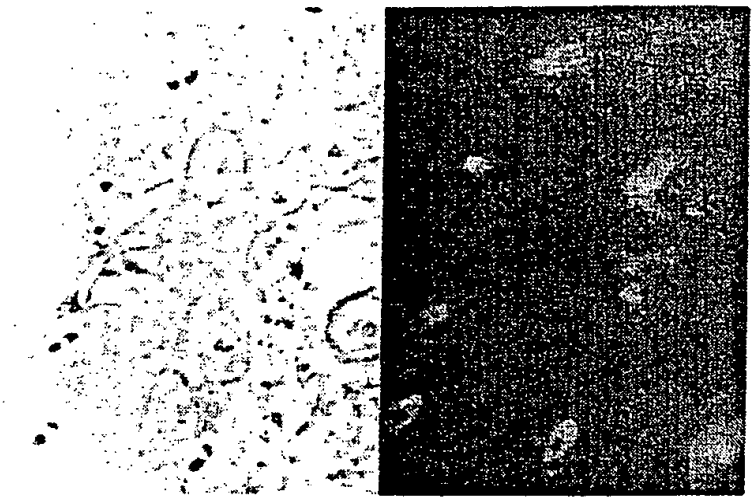
7B2 3 hrs



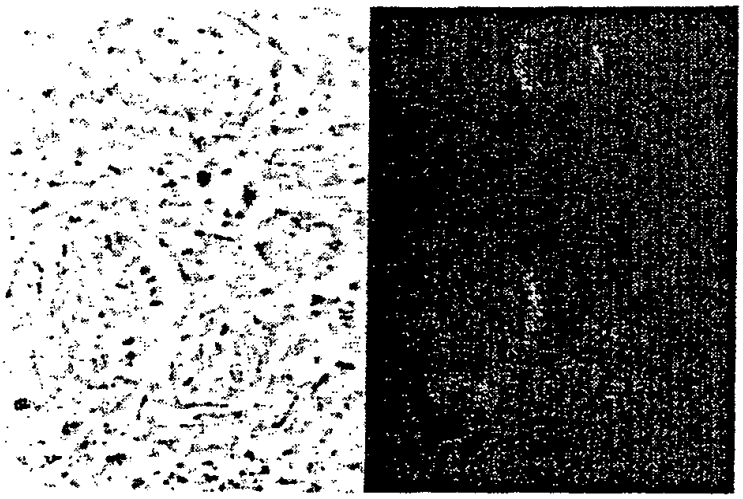
15A3 19 hrs

Fig. 8A

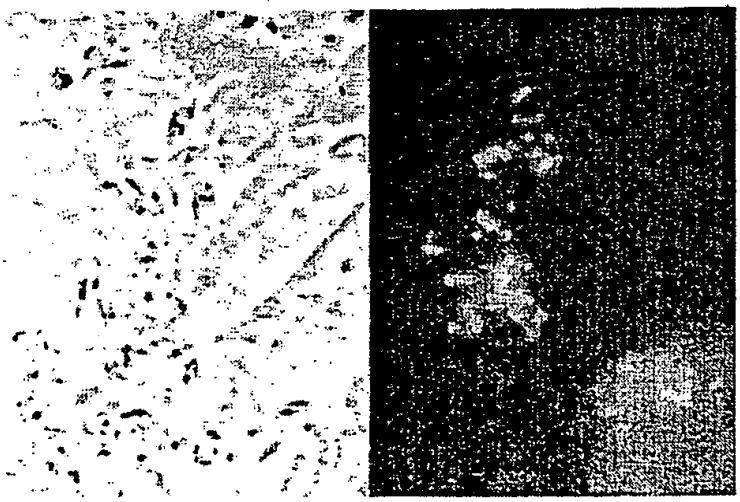
14B1



16 hrs

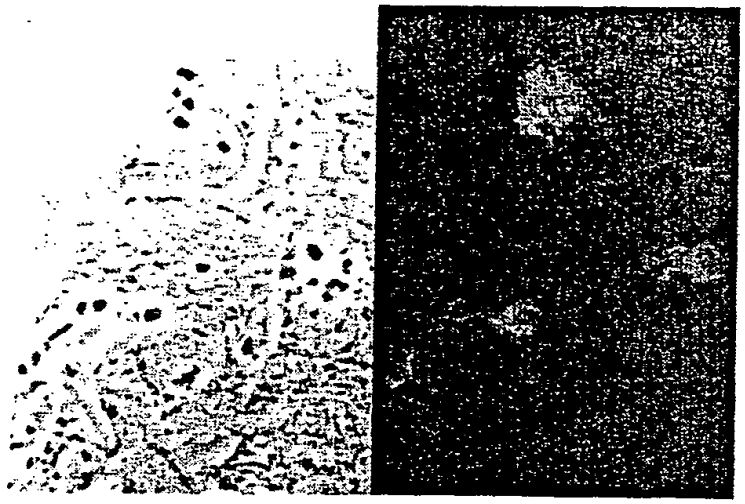


100 hrs



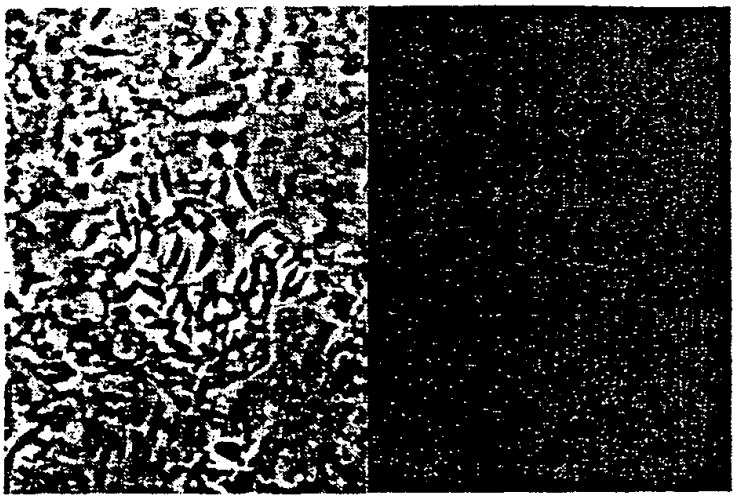
120 hrs

Fig. 8B



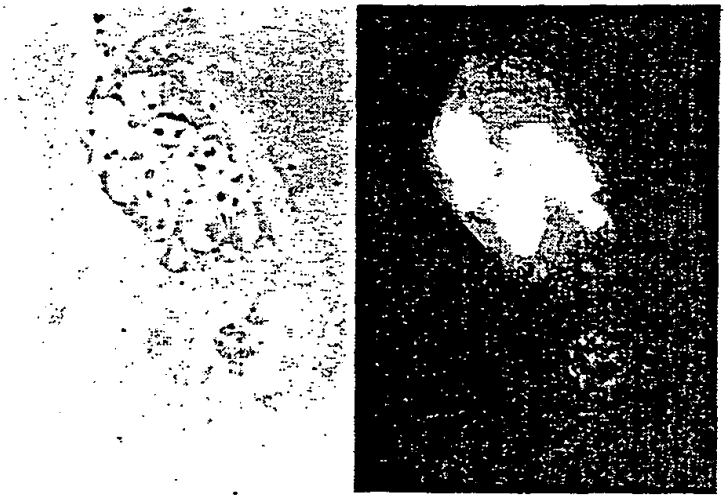
19D6

3 hrs



19D6

120 hrs



immune

chick sera

3 hrs

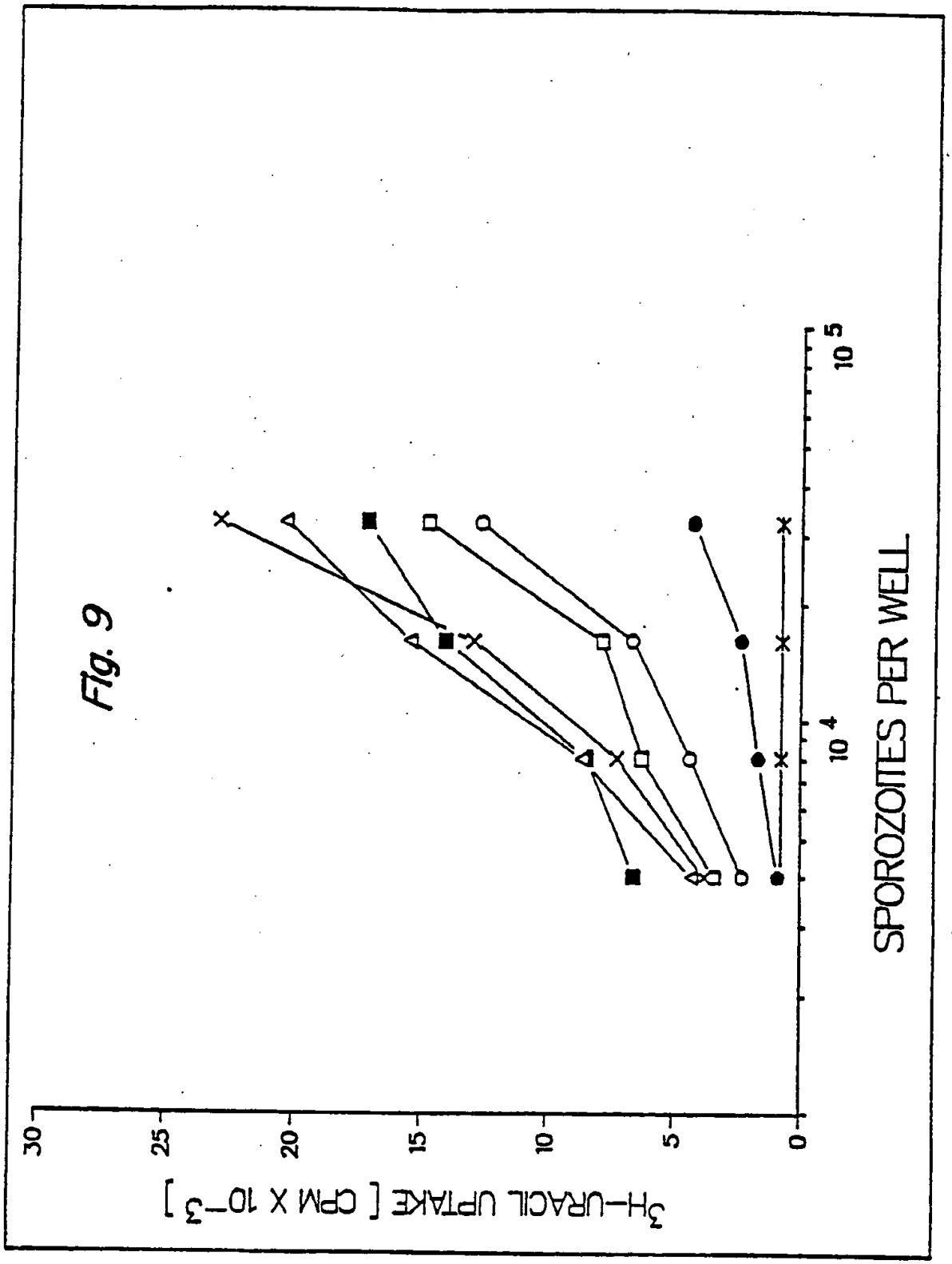

^{3}H–URACIL UPTAKE [CPM X 10^{-3}]
30
25
20
15
10
5
0
10^4
10^5
SPOROZOITES PER WELL

Fig. 9

Fig. 10

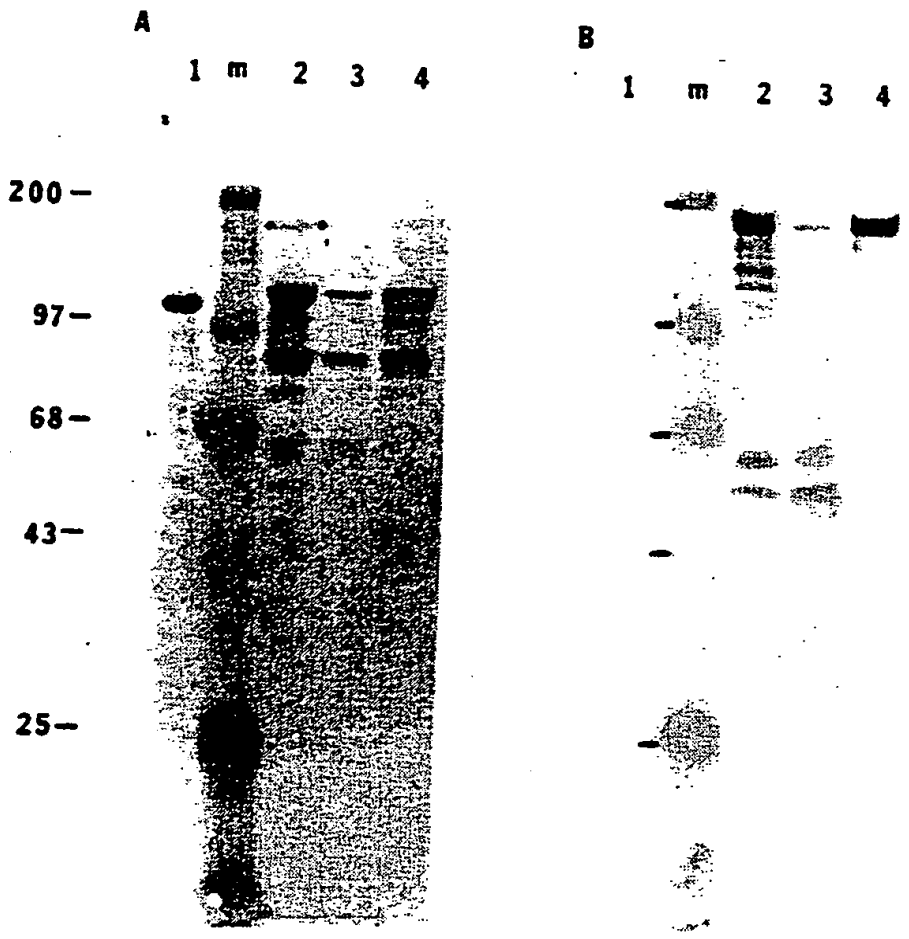


Fig. 11

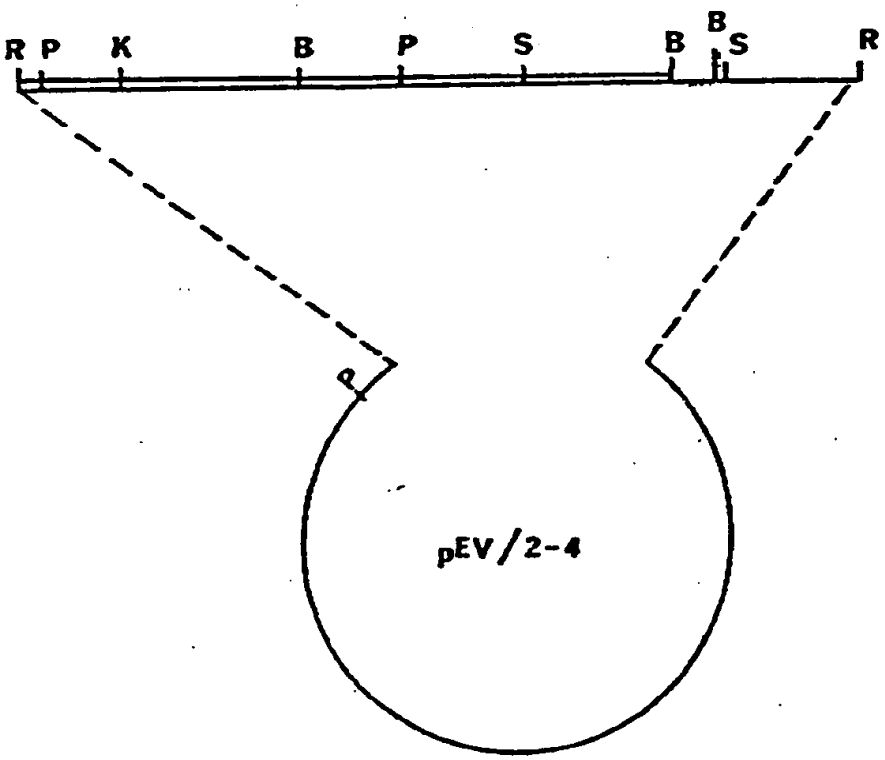


Fig. 12

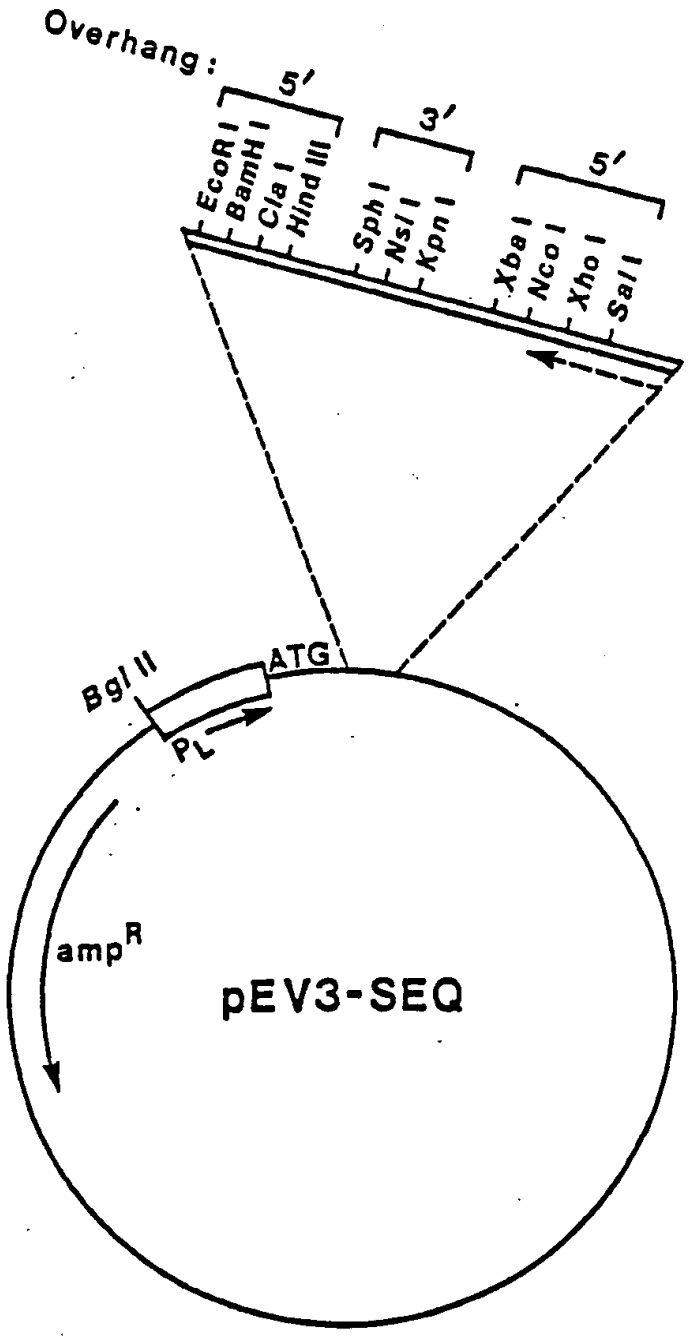

Overhang :
5'
3'
5'
EcoR I
BamH I
Cla I
Hind III
Sph I
Nsi I
Kpn I
Xba I
Nco I
Xho I
Sal I
Bgl II
ATG
PL
amp^R
pEV3-SEQ

Fig. 13

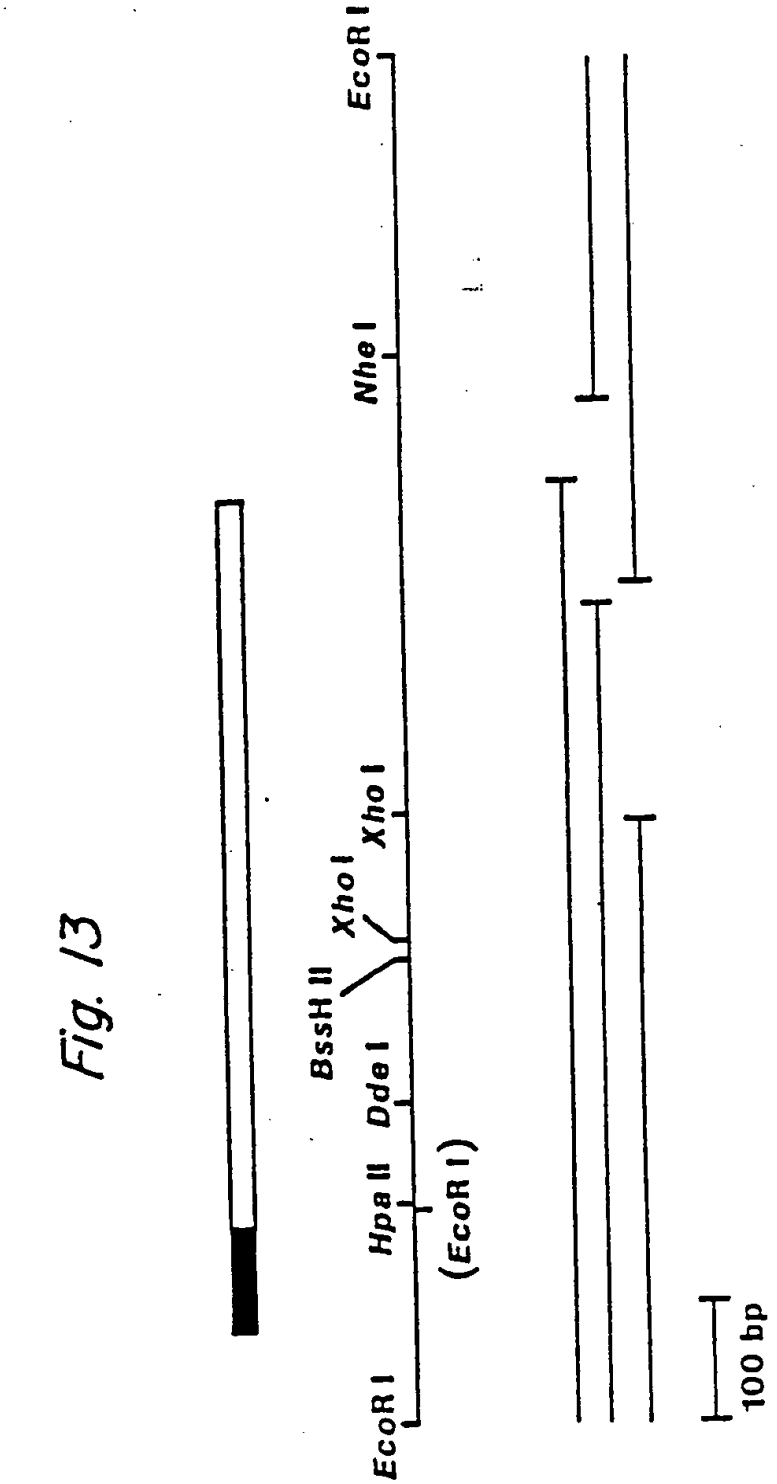


Fig. 14A

```
        10         20         30         40         50
GAATTCCCTC CAACTCTTCG CGACTCTCTC TCTCTGCCCC CAACTTTTTC

        60         70         80         90        100
CCCCGCGCCC CGCAGCAGCA GCAGCAGCAG CAGCAGCAAA ATGGCAGACC

       110        120        130        140        150
TCTTCAGCGG ACTCGTGGGC GGCGTCGTCG GCGCTGTTGC TGCAGCAGAT

       160        170        180        190        200
TTGCCTGCGG AGGGCGAGAG GGCCCCCCGC CCCGCCCCCG GCACTGCCTG

       210        220        230        240        250
GACTTGCTGC TGCAGCAAAC TGCAAGAAGG GGCCCGCGAG CTGGAGGGTT

       260        270        280        290        300
TTGTGCAGCA GCTGAGTTTT GTTGCAGGGA AGCTGGCCTG CTGCCTGCGG

       310        320        330        340        350
GTGGGGGCGG AGCAGCTGGC GCGCTGCGCT GCGGAGGGGC GGCTGCCCAG

       360        370        380        390        400
CAGCAGCAGC AGCAGCAGCT GCTGCGCGCT GCTGCAGCTC GAGAAGCAGG

       410        420        430        440        450
ACCTCGAGCA GAGCCTCGAG GCCGGCAAGC AGGGCGCGGA GTGCCTCTTG

       460        470        480        490        500
AGGAGCAGCA AACTGGCCCT CGAGGCCCTC CTCGAGGGGG CCCGCGTTGC

       510        520        530        540        550
AGCAACGCGG GGTTTGCTGC TGGTCGAGAG CAGCAAAGAC ACGGTGCTGC

       560        570        580        590        600
GCAGCATTCC CCACACCCAG GAGAAGCTGG CTCAGGCCTA CAGTTCTTTC

       610        620        630        640        650
CTGCGGGGCT ACCAGGGGGC AGCAGCGGGG AGGTCTCTGG GCTACGGGGC

       660        670        680        690        700
CCCTGCTGCT GCTTACGGCC AGCAGCAGCA GCCCAGCAGC TACGGGGCGC

       710        720        730        740        750
CCCCCGCCTC CAGCCAGCAG CCCTCCGGCT TCTTCTGGTA GCCCTGCAGC

       760        770        780        790        800
AGCAGCAGCA GCAGCAGCAG CAGCAGCAGC GGCGGCGGCA GCCGCGGCGG

       810        820        830        840        850
GGCCGGGGCG CCGCTGCAGC AACAGCAGCA GCCGCGGCGG CTAGCG3636

       860        870        880        890        900
GAGCACTCGC AGGGAACTCC ACAGGCAGCG GGAGAGCAGC AGGGACGAGA
```

Fig. 14B

```
      910        920        930        940        950
AGCAGGTCTA TGTAGCGCAG GCAGCAGCGC CAGCTGCAGC AGCAGCAGCA

      960        970        980        990       1000
GCAGCAGCAG CAGCAGCAGC AGCTCCTGCA CCGCAGCGTT GTGTCATTTA

     1010       1020       1030       1040       1050
TTACGTTGGC AGCTCTGAGG CCTCGGCGCA GCCAACGCGC CTCAGGTATC

     1060       1070       1080
TTTCAGACTC TTTTCTCTAA GGTCTTCCAG ACGGAATTC
```

Fig. 15

```
                                                  10                                                  20
Met Ala Asp Leu Phe Ser Gly Leu Val Gly Gly Val Val Gly Ala Val Ala Ala Ala Asp

                                                  30                                                  40
Leu Pro Ala Glu Gly Glu Arg Ala Pro Arg Pro Ala Pro Gly Thr Ala Trp Thr Cys Cys

                                                  50                                                  60
Cys Ser Lys Leu Gln Glu Gly Ala Arg Glu Leu Glu Gly Phe Val Gln Gln Leu Ser Phe

                                                  70                                                  80
Val Ala Gly Lys Leu Ala Cys Cys Leu Arg Val Gly Ala Glu Gln Leu Ala Arg Cys Ala

                                                  90                                                 100
Ala Glu Gly Arg Leu Pro Ser Ser Ser Ser Ser Ser Ser Cys Cys Ala Leu Leu Gln Leu

                                                 110                                                 120
Glu Lys Gln Asp Leu Glu Gln Ser Leu Glu Ala Gly Lys Gln Gly Ala Glu Cys Leu Leu

                                                 130                                                 140
Arg Ser Ser Lys Leu Ala Leu Glu Ala Leu Leu Glu Gly Ala Arg Val Ala Ala Thr Arg

                                                 150                                                 160
Gly Leu Leu Leu Val Glu Ser Ser Lys Asp Thr Val Leu Arg Ser Ile Pro His Thr Gln

                                                 170                                                 180
Glu Lys Leu Ala Gln Ala Tyr Ser Ser Phe Leu Arg Gly Tyr Gln Gly Ala Ala Ala Gly

                                                 190                                                 200
Arg Ser Leu Gly Tyr Gly Ala Pro Ala Ala Ala Tyr Gly Gln Gln Gln Gln Pro Ser Ser

                                                 210                 216
Tyr Gly Ala Pro Pro Ala Ser Ser Gln Gln Pro Ser Gly Phe Phe Trp
```

EP 0 344 808 A1

Fig. 16

```
   1 GAATTCATGT TTAGGCGGAT TTTGTTCCAT GCAAGAAAGC CCACCACCGG CTGCAGGTGG
  61 ACTGTACGGG GGACAGACTT TGGAACAACA AGGCATTGCT GTGAGGGAAA CTGCTTCGTG
 121 CAGCGAGAAC CCGTGCCCTA TCGACGCAAC GTGCGGAGAA TGGACAGAGT ACAGTGCGTG
 181 CTCCAGAACT TGCGGAGGCG GTACCCAAGA GAGGAAGAGG GAGCCGTGGT TGGATAATGC
 241 GCAACACGGG GGGCGCACCT GCATGGAACA GTATCCTGAT GGGCCCATAT CGGTCAGGGA
 301 GTGCAACACC CAGCCGTGCC CTGTGGACGA AGTAGTTGGT GATTGGGAAG ACTGGGGGCA
 361 ATGCAGCGAA CAGTGTGGTG GCGGCAAGCG GACTCGTAAT CGCGGCCCAA GCAAGCAAGA
 421 GGCCATGTTC GGAGGCAAGA CAGTTGCTCA ACAGAACGCA GAGCTCCCTG AAGGCGAGAA
 481 GATTGAGGTG GTTCAGGAAG AAGGATGCAA TGAAGTTCCA TGCGGACCTT GCACGCTCCC
 541 CTTCAGTGAG TGGACCGAAT GCGAGTCGTG CTCCGGGCAT AGAACCAGGG AATCCGCAGT
 601 AGCATTTGAT TACACTGACA GAATGTGCAG TGGTGACACA CACGAGGTAC AAAGCTGTGA
 661 GGAATACTGT TCCCAAAATG CTGGAGGGGG TGCTGGAGGA GATGGGGGCG CAGGAGGAGG
 721 GACTGGAGGC TCTGGAGAGG AGGAAGGAAA GGAGGAATCG AGTGGATTTC CAACTGCAGC
 781 TGTAGCCGGT GGCGTGGCTG GGGGAGTCCT CGCGATTGCT GCGGGAGCTG GAGCGTTTTA
 841 TGGATTGAGT GGTGGGAGCG CGGCTGCTGC CACTGAAGCA GGTGCTGAAG TGATGACAGA
 901 AGCTGGTACA TCCAATGCTG CTGAGGTAGA AAAGGAGAGC CTCATCAGTG CAGGTGAACA
 961 ATCAGAGATG TGGGCATCCT AAATGGAAAC GTCGCCGCCG CGGGTTTCGA AAAGGTGCGG
1021 ATCTTGCATA TCTGTGAACG AATTATTTAC TAACATCGAG CTCCTTGACC TCCCGTTGGC
1081 AAATCATTTA CCAAGCATCT CTGGCGCATA GCTTCTTGAA CAAGACAACG GAATGTCCAA
1141 CTGGGGAACA GCTATATTGC GAAGTGTGGT GTTCAAACCA GAAGAGAGCA CAGCGTCATG
1201 TGTATGTTAG GGTTGGGCGC CTCCTTTCCC TTATTTATCC CATTTCCTCC GCCTTCATCT
1261 TTCCGCCTTC TCTCTGTGCG CCGTATTTTG GGTGTTATTG GTGCCTGGCG GACAGTAAAG
1321 AGAGATTGGC GTTATTTGCA GCGTGCGCAG GCCATGGTAG GGTTGGATAA CACTCATTGG
1381 TGAAGCGCAA GCCAACAGGG CCACCTTTAC CTCCTGGTGG TCAATGGGGC AGCTTGCTTC
1441 TGATCATTGG TTGGTTCTGT TTCAAGGGGC CGGTAATGGG CAGCAGAAGC TTCTGCCAGC
1501 CACCACACAA TCGAAGCAAC AAATAAGGGA GGTTCTGCTA ACAATTGTGC GTAGTCATGA
1561 TTGTAGGTAG GCTCCGTTTC GAAGATGAAT GACCGGGAGC AGCCTGAATG AAACTTGACT
1621 CTCAAAGAAG GGAATTC
```

Fig. 18A

```
        10         20         30         40         50
GAATTCCCGA CTTCGAGGGA GGCCCCGGGG GCCTCCCCGC CTGCGAAGCG

        60         70         80         90        100
GCGGCGGACG TCGCTGGGGG CCCCTGCAGC AGGGGAGGGC CCCCTGCGGC

       110        120        130        140        150
GGTGGGAGCA GCCTGCTGCT GGGACTGCAG CAGCAATCCG GCAGCAGGCT

       160        170        180        190        200
GGAGGAGCGG GAGCAGCAGC GGCAGCGCGA GCGGCAGCTG CAGCACGTGC

       210        220        230        240        250
GCGCACCCCC GGGCGCGCAG CAGCAGTGCA GGCGCGGCTA AACGCGTGGG

       260        270        280        290        300
TGGCGGAGGG CAACAAACTG CCAGAGTCCG AGAGAAGAAG AAGGATGTTA

       310        320        330        340        350
GAACAATATA TGAACTTGGA AAAAGTCAAA AAGTTGAGGA AAAAACTCGA

       360        370        380        390        400
CGAAGAAGCA GAAGCCAGGG CCAAATACAT CGAAGGCGGA GTTCAAAAAG

       410        420        430        440        450
AACCCCCACT GGGGGCCCCT CAAGGCCGAA AACCCTTTGC TGCCTTTTGC

       460        470        480        490        500
CCAGAGAGAG GCCGACGAGG CCTACAGGCG GTTCGGCAGG GGCGCTCCCT

       510        520        530        540        550
CTGCGGGGCC CCTCAGGGAG AAGATGCTGC AGGCCCGCAG GAAGTAAAGC

       560        570        580        590        600
AGCAGCAGCA GCAGCAGCAG CAGCGGCAGC GGCAGCGGCA GGGGAGGCGG

       610        620        630        640        650
AGGAGGCAGG GAGGCTTTTG TTTCGAATTG TTTAGGGAGA GGGCGGAGGG

       660        670        680        690        700
CTCGCGGGGT GTATGTACAG CGCGCGAACG CGGCGGCTCA TGTTTGGGTG

       710        720        730        740        750
TTGGATTTCG TCTGCAGAAA ACACGAAGCA AATTAAACTG GCAGAAGTTC

       760        770        780        790        800
CATTTTTCCA CTTTAAAATG CCACTTTTGC TCTTTATATT GATTAAAACT

       810        820        830        840        850
AATGCCATGC TGCTGGCATT CATATAAATG CATTTCCGCG CATTTATGTG

       860        870        880        890        900
CATGCATGGC TGGGCGCGGG TGTCTGTACA CCCCAAGAGC CTTGCTGCTC
```

Fig. 18B

```
      910        920        930        940        950
CGCCGGCGCG AATTTATATT TATATTTCAT TTATGTAAAT ATAAAAGCCT

      960        970        980        990       1000
TCAAAAACAC AAATGGACAT TAATTTATCA AGAAAAAAGA TTAAGGAATT C
```

Fig. 19

```
                                                  10                  20
Glu Phe Pro Thr Ser Arg Glu Ala Pro Gly Ala Ser Pro Pro Ala Lys Arg Arg Arg Thr

                                                  30                  40
Ser Leu Gly Ala Pro Ala Ala Gly Glu Gly Pro Leu Arg Arg Trp Glu Gln Pro Ala Ala

                                                  50                  60
Gly Thr Ala Ala Ala Ile Arg Gln Gln Ala Gly Gly Ala Gly Ala Ala Ala Ala Ala Arg

                                                  70                  80
Ala Ala Ala Ala Ala Arg Ala Arg Thr Pro Gly Arg Ala Ala Ala Val Gln Ala Arg Leu

                                                  90                 100
Asn Ala Trp Val Ala Glu Gly Asn Lys Leu Pro Glu Ser Glu Arg Arg Arg Arg Met Leu

                                                 110                 120
Glu Gln Tyr Met Asn Leu Glu Lys Val Lys Lys Leu Arg Lys Lys Leu Asp Glu Glu Ala

                                                 130                 140
Glu Ala Arg Ala Lys Tyr Ile Glu Gly Gly Val Gln Lys Glu Pro Pro Leu Gly Ala Pro

                                                 150                 160
Gln Gly Arg Lys Pro Phe Ala Ala Phe Cys Pro Glu Arg Gly Arg Arg Gly Leu Gln Ala

                                                 170                 180
Val Arg Gln Gly Arg Ser Leu Cys Gly Ala Pro Gln Gly Glu Asp Ala Ala Gly Pro Gln

                                                 190                 200
Glu Val Lys Gln Gln Gln Gln Gln Gln Gln Gln Arg Gln Arg Gln Arg Gln Gly Arg Arg

                                                 210                 220
Arg Arg Gln Gly Gly Phe Cys Phe Glu Leu Phe Arg Glu Arg Ala Glu Gly Ser Arg Gly

                                                 230                 240
Val Cys Thr Ala Arg Glu Arg Gly Gly Ser Cys Leu Gly Val Gly Phe Arg Leu Gln Lys

                                                 250                 260
Thr Arg Ser Lys Leu Asn Trp Gln Lys Phe His Phe Ser Thr Leu Lys Cys His Phe Cys

Ser Leu Tyr
```

Fig. 20A

```
        10         20         30         40         50
GAATTCCGCC CCAGACAGCT AAGCGTGGCA ACATTCTTGG TCTTGTGGGC

        60         70         80         90        100
ATGGTAGCCG CTGTCGTCGT GACCTTCACG GAGGCAGGGT TTGGACAGCA

       110        120        130        140        150
TTACTTGCTG TTTTTCGCCA CTGCTGCACC GGCCCTTGGC CTGGGGCTGT

       160        170        180        190        200
ACATTGCGCA GTCTGTCAAC ATGACTGAGA TGCCTCAACT CGTGGCTCTT

       210        220        230        240        250
TTCCACAGTT TCGTCGGTCT TGCCGCCGTA ATGGTTGGGT TCGCGAACTT

       260        270        280        290        300
CCACTCCCCT GCTGGCGTGG AGCGCGCTTC CTCACTTCTA CGTCTGTTGG

       310        320        330        340        350
AGGTCTACGC CGGCGTTTTC GTCGCCGGTA TCACCTTCAC CGGATCAGTG

       360        370        380        390        400
GTCGCTGCGG CAAAGCTCCA TGGATCGATG GAGAGCCGCT CATTGAGGGT

       410        420        430        440        450
TCCCGGACGC CATGCGTTGA ATACTGCCAC TATTGCTGCC ATTGGCGTAC

       460        470        480        490        500
TTGGCGCTCT TTTTTGCGTC TCTTCTGGCC ACTTTACACG CATGCTTTGC

       510        520        530        540        550
CTTTATGTGA ATGCTGGCTT GAGCATGTGG CTTGGTTTTC ACCTGGTCGC

       560        570        580        590        600
CGCTATTGGT GGAGCTGACA TGCCCGTCGT GATCAGCTTG CTGAACTCGT

       610        620        630        640        650
ATTCCGGAGT GGCGTTGGCT GCCAGTGGCT TCATGTTGGA CAACAACTTG

       660        670        680        690        700
CTGATCATTG CTGGTGCTCT CATCGCGTCA TCTGGTGCCA TTCTGTCTTA

       710        720        730        740        750
CATCATGTGC AAAGGCATGA ACCGGAGTCT GTGGAATGTC GTTCTTGGTG

       760        770        780        790        800
GCTTCGAGGA GGCCGAGGAC GTTGGCGCAG CCAGCCCTCA GGGGGCTGTG

       810        820        830        840        850
CAGCAGGCCA CGGCTGATCA GGTCGCCGAC GAGTTGCTGG CTGCCCGCAA

       860        870        880        890        900
AGTTTTGATC GTGCCTGGAT ACGGAATGGC CGTTGCAAGG TGCCAGAGCG
```

Fig. 20B

```
      910         920         930         940         950
AGCTTGCAGA CATTGCCAAG AACTTGATGA ACTGCGGTAT CACCGTCGAT

      960         970         980         990        1000
TTCGGCATCC ATCCAGTTGC TGGTCGCATG CCAGGCCACA TGAACGTCCT

     1010        1020        1030        1040        1050
CCTCGCTGAG GCTGATGTTC CGTACAAGAT TGTCAAGGAG ATGTCTGAAG

     1060        1070        1080        1090        1100
TCAACCCGGA AATGAGCTCC TACGACGTCG TCCTGGTTGT TGGAGCCAAC

     1110        1120        1130        1140        1150
GACACCGTCA ATCCTGCAGC CCTTGAGCCA GGATCAAAGA TCTCAGGAAT

     1160        1170        1180        1190        1200
GCCCGTTATA GAGGCCTGGA AAGCTAGACG CGTTTTTGTG CTGAAGCGGT

     1210        1220        1230        1240        1250
CCATGGCTGC TGGATATGCC AGCATTGAAA ATCCACTTTT CCATCTGGAG

     1260        1270        1280        1290        1300
AACACACGCA TGCTCTTCGG AAACGCAAAG AACACCACTT CTGCAGTCTT

     1310        1320        1330        1340        1350
CGCCCGTGTC AATGCCAGAG CCGAGCAAAT GCCACCATCT GCTGCCCGTG

     1360        1370        1380        1390        1400
ATGACCTCGA AGCTGGACTA CTTGAGTTCG ATAGGGAAGA ACGTGTTGAT

     1410        1420        1430        1440        1450
CCCTCTTCTT GGCCATATCC CAGGATGGCT GTTGGTGTTC TGAGAGACTC

     1460        1470        1480        1490        1500
CAATGGCTCT GTTATGGTGC CAGTAGCTCC GAAGTTTGTG CCCAAGCTGA

     1510        1520        1530        1540        1550
GGAAGTTGGC ATTCCGTGTC AATGTCGAGT CTGGTGCTGG CGCCGATGCC

     1560        1570        1580        1590        1600
GGCTTTACTG ACGAAGAGTA CAGGAGGGCT GGAGCAGAAG TCCTGTCGGG

     1610        1620        1630        1640        1650
CCCCGATGCA GTCATTAACC AGTCTCAAGT CCTGCTCCGC GTTTCAGCGC

     1660        1670        1680        1690        1700
CGTCGCCAGA TCTGGTTTCG CGCATTCCTA GGGACAAGGT CCTTATCAGT

     1710        1720        1730        1740        1750
TACCTATTCC CCAGCATCAA CCAACAAGCT CTTGACATGC TAGCACGCCA

     1760        1770        1780        1790        1800
AGGCGTCACC GCACTTGCTG TGGATGAGGT TCCTCGCGTC ACAAGAGCAC
```

Fig. 20C

```
      1810        1820        1830        1840        1850
AGAAGCTAGA CGTGAAGTCT GCTATGCAAG GTCTCCAGGG ATACCGCGCC

      1860        1870        1880        1890        1900
GTTATCGAAG CGTTCAACGC TCTTCCGAAG CTCAGCAAAG CATCTATCAG

      1910        1920        1930        1940        1950
TGCTGCAGGC CGTGTAGAAG CCGCTAAAGT TTTCGTCATC GGTGCCGGTG

      1960        1970        1980        1990        2000
TTGCTGGACT ACAGGCAATT TCTACCGCCC ATGGTTTGGG TGCACAAGTA

      2010        2020        2030        2040        2050
GTTGGCCACG ATGTGCGCTC TGCAACTCGT GAGGAAGTCG AATCTTGCGG

      2060        2070        2080        2090        2100
TGGAAAGTTC ATTGGTTTGA GAATGGGAGA GGAGGGTGAA GTCCTCGGAG

      2110        2120        2130        2140        2150
GATATGCACG CGAGATGGGT GATGCATATC AGAGAGCGCA ACGCGAGATG

      2160        2170        2180        2190        2200
ATTGCCAACA CAATCAAGCA CTGCGATGTC GTCATCTGTA CCGCTGCTAT

      2210        2220        2230        2240        2250
TCACGGCAGA CCTTCACCAA AGCTCATATC ACGCGACATG TTGCGTTCAA

      2260        2270        2280        2290        2300
TGAAGCCTGG CTCCGTCGTC GTAGATCTTG CAACAGAGTT CGGTGATGTG

      2310        2320        2330        2340        2350
CGCTCCGGCT GGGGTGGAAA CGTCGAGGTT TCGCCTAAGG ACGACCAGAT

      2360        2370        2380        2390        2400
TGTCGTTGAT GGCGTCACTG TCATTGGTCG CAGACGCATT GAGACTCGCA

      2410        2420        2430        2440        2450
TGCCCATTCA GGCGTCTGAG CTGTTCTCCA TGAACATATG CAACCTCCTT

      2460        2470        2480        2490        2500
GAGGATCTTG GTGGTGGCAG CAACTTCCGC ATCAACATGG ACGACGAAGT

      2510        2520        2530        2540        2550
CATCAGAGGA TTGGTCGCAG TCTACCAAGG TCGCAACGTG TGGCAGCCAT

      2560        2570        2580        2590        2600
CGCAGCCCAC TCCTGTTTCC AGGACACCTC CGCGCGGCCA GATGCCGCCC

      2610        2620        2630        2640        2650
CCGTCTGCAC CTGGTGCACC AGCTCCTGAG AAGCCTGGTG CCTTTGCTCA

      2660        2670        2680        2690        2700
AGCACTTGCT TCGGATGCAT TCTTCGCAAT GTGTCTTGTT GTTGCTGCCG
```

Fig. 20D

```
      2710        2720        2730        2740        2750
CTGTTGTCGG GCTCCTTGGC ATTGTCCTTG ACCCTGTGGA GCTCAAGCAT

      2760        2770        2780        2790        2800
TTGACTCTCC TCGGCTTGTC TCTCATCGTC GGCTACTACT GCGTGTGGGC

      2810        2820        2830        2840        2850
CGTTACGCCT TCGCTTCACA CACCATTGAT GTCTGTGACG AATGCCCTTT

      2860        2870        2880        2890        2900
CGGGAGTCAT TGTCATCGGC TGCATGCTCG AGTACGGAAC CGCCATGATA

      2910        2920        2930        2940        2950
TCCGGATTCA CTCTTCTCGC ACTCATTGGA ACCTTCTTGG CTTCCGTCAA

      2960        2970        2980        2990        3000
CGTTGCTGGT GGATTCTTCG TAACTCACCG CATGCTGAAG ATGTTTCAGA

      3010        3020        3030        3040        3050
TATAAGGGGA GAACCCCCTT GAGTTAATCT TAACTCAGAA TAACTCTTTT

      3060        3070        3080        3090
TCAATTGTAT AAACCTGTAC TCGTTGCAAA AAAAAAAGGA ATTC
```

Fig. 21A

```
                                   10                                  20
Pro Gln Thr Ala Lys Arg Gly Asn Ile Leu Gly Leu Val Gly Met Val Ala Ala Val Val

                                   30                                  40
Val Thr Phe Thr Glu Ala Gly Phe Gly Gln His Tyr Leu Leu Phe Phe Ala Thr Ala Ala

                                   50                                  60
Pro Ala Leu Gly Leu Gly Leu Tyr Ile Ala Gln Ser Val Asn Met Thr Glu Met Pro Gln

                                   70                                  80
Leu Val Ala Leu Phe His Ser Phe Val Gly Leu Ala Ala Val Met Val Gly Phe Ala Asn

                                   90                                 100
Phe His Ser Pro Ala Gly Val Glu Arg Ala Ser Ser Leu Leu Arg Leu Leu Glu Val Tyr

                                  110                                 120
Ala Gly Val Phe Val Ala Gly Ile Thr Phe Thr Gly Ser Val Val Ala Ala Ala Lys Leu

                                  130                                 140
His Gly Ser Met Glu Ser Arg Ser Leu Arg Val Pro Gly Arg His Ala Leu Asn Thr Ala

                                  150                                 160
Thr Ile Ala Ala Ile Gly Val Leu Gly Ala Leu Phe Cys Val Ser Ser Gly His Phe Thr

                                  170                                 180
Arg Met Leu Cys Leu Tyr Val Asn Ala Gly Leu Ser Met Trp Leu Gly Phe His Leu Val

                                  190                                 200
Ala Ala Ile Gly Gly Ala Asp Met Pro Val Val Ile Ser Leu Leu Asn Ser Tyr Ser Gly

                                  210                                 220
Val Ala Leu Ala Ala Ser Gly Phe Met Leu Asp Asn Asn Leu Leu Ile Ile Ala Gly Ala

                                  230                                 240
Leu Ile Ala Ser Ser Gly Ala Ile Leu Ser Tyr Ile Met Cys Lys Gly Met Asn Arg Ser

                                  250                                 260
Leu Trp Asn Val Val Leu Gly Gly Phe Glu Glu Ala Glu Asp Val Gly Ala Ala Ser Pro

                                  270                                 280
Gln Gly Ala Val Gln Gln Ala Thr Ala Asp Gln Val Ala Asp Glu Leu Leu Ala Ala Arg

                                  290                                 300
Lys Val Leu Ile Val Pro Gly Tyr Gly Met Ala Val Ala Arg Cys Gln Ser Glu Leu Ala

                                  310                                 320
Asp Ile Ala Lys Asn Leu Met Asn Cys Gly Ile Thr Val Asp Phe Gly Ile His Pro Val

                                  330                                 340
Ala Gly Arg Met Pro Gly His Met Asn Val Leu Leu Ala Glu Ala Asp Val Pro Tyr Lys
```

Fig. 2B

```
                                                350                                                360
Ile Val Lys Glu Met Ser Glu Val Asn Pro Glu Met Ser Ser Tyr Asp Val Val Leu Val

                                                370                                                380
Val Gly Ala Asn Asp Thr Val Asn Pro Ala Ala Leu Glu Pro Gly Ser Lys Ile Ser Gly

                                                390                                                400
Met Pro Val Ile Glu Ala Trp Lys Ala Arg Arg Val Phe Val Leu Lys Arg Ser Met Ala

                                                410                                                420
Ala Gly Tyr Ala Ser Ile Glu Asn Pro Leu Phe His Leu Glu Asn Thr Arg Met Leu Phe

                                                430                                                440
Gly Asn Ala Lys Asn Thr Thr Ser Ala Val Phe Ala Arg Val Asn Ala Arg Ala Glu Gln

                                                450                                                460
Met Pro Pro Ser Ala Ala Arg Asp Asp Leu Glu Ala Gly Leu Leu Glu Phe Asp Arg Glu

                                                470                                                480
Glu Arg Val Asp Pro Ser Ser Trp Pro Tyr Pro Arg Met Ala Val Gly Val Leu Arg Asp

                                                490                                                500
Ser Asn Gly Ser Val Met Val Pro Val Ala Pro Lys Phe Val Pro Lys Leu Arg Lys Leu

                                                510                                                520
Ala Phe Arg Val Asn Val Glu Ser Gly Ala Gly Ala Asp Ala Gly Phe Thr Asp Glu Glu

                                                530                                                540
Tyr Arg Arg Ala Gly Ala Glu Val Leu Ser Gly Pro Asp Ala Val Ile Asn Gln Ser Gln

                                                550                                                560
Val Leu Leu Arg Val Ser Ala Pro Ser Pro Asp Leu Val Ser Arg Ile Pro Arg Asp Lys

                                                570                                                580
Val Leu Ile Ser Tyr Leu Phe Pro Ser Ile Asn Gln Gln Ala Leu Asp Met Leu Ala Arg

                                                590                                                600
Gln Gly Val Thr Ala Leu Ala Val Asp Glu Val Pro Arg Val Thr Arg Ala Gln Lys Leu

                                                610                                                620
Asp Val Lys Ser Ala Met Gln Gly Leu Gln Gly Tyr Arg Ala Val Ile Glu Ala Phe Asn

                                                630                                                640
Ala Leu Pro Lys Leu Ser Lys Ala Ser Ile Ser Ala Ala Gly Arg Val Glu Ala Ala Lys

                                                650                                                660
Val Phe Val Ile Gly Ala Gly Val Ala Gly Leu Gln Ala Ile Ser Thr Ala His Gly Leu

                                                670                                                680
Gly Ala Gln Val ??? Gly His Asp Val Arg Ser Ala Thr Arg Glu Glu Val Glu Ser Cys

                                                690                                                700
Gly Gly Lys Phe Ile Gly Leu Arg Met Gly Glu Glu Gly Glu Val Leu Gly Gly Tyr Ala
```

Fig. 2IC

```
                                                                710
Arg Glu Met Gly Asp Ala Tyr Gln Arg Ala Gln Arg Glu Met Ile Ala Asn Thr Ile Lys
                                                                730
His Cys Asp Val Val Ile Cys Thr Ala Ala Ile His Gly Arg Pro Ser Pro Lys Leu Ile
                                                                750
Ser Arg Asp Met Leu Arg Ser Met Lys Pro Gly Ser Val Val Val Asp Leu Ala Thr Glu
                                                                770
Phe Gly Asp Val Arg Ser Gly Trp Gly Gly Asn Val Glu Val Ser Pro Lys Asp Asp Gln
                                                                790
Ile Val Val Asp Gly Val Thr Val Ile Gly Arg Arg Arg Ile Glu Thr Arg Met Pro Ile
                                                                810
Gln Ala Ser Glu Leu Phe Ser Met Asn Ile Cys Asn Leu Leu Glu Asp Leu Gly Gly Gly
                                                                830
Ser Asn Phe Arg Ile Asn Met Asp Asp Glu Val Ile Arg Gly Leu Val Ala Val Tyr Gln
                                                                850
Gly Arg Asn Val Trp Gln Pro Ser Gln Pro Thr Pro Val Ser Arg Thr Pro Pro Arg Gly
                                                                870
Gln Met Pro Pro Pro Ser Ala Pro Gly Ala Pro Ala Pro Glu Lys Pro Gly Ala Phe Ala
                                                                890
Gln Ala Leu Ala Ser Asp Ala Phe Phe Ala Met Cys Leu Val Val Ala Ala Ala Val Val
                                                                910
Gly Leu Leu Gly Ile Val Leu Asp Pro Val Glu Leu Lys His Leu Thr Leu Leu Gly Leu
                                                                930
Ser Leu Ile Val Gly Tyr Tyr Cys Val Trp Ala Val Thr Pro Ser Leu His Thr Pro Leu
                                                                950
Met Ser Val Thr Asn Ala Leu Ser Gly Val Ile Val Ile Gly Cys Met Leu Glu Tyr Gly
                                                                970
Thr Ala Met Ile Ser Gly Phe Thr Leu Leu Ala Leu Ile Gly Thr Phe Leu Ala Ser Val
                                                                990                998
Asn Val Ala Gly Gly Phe Phe Val Thr His Arg Met Leu Lys Met Phe Gln Ile
```

Fig. 22

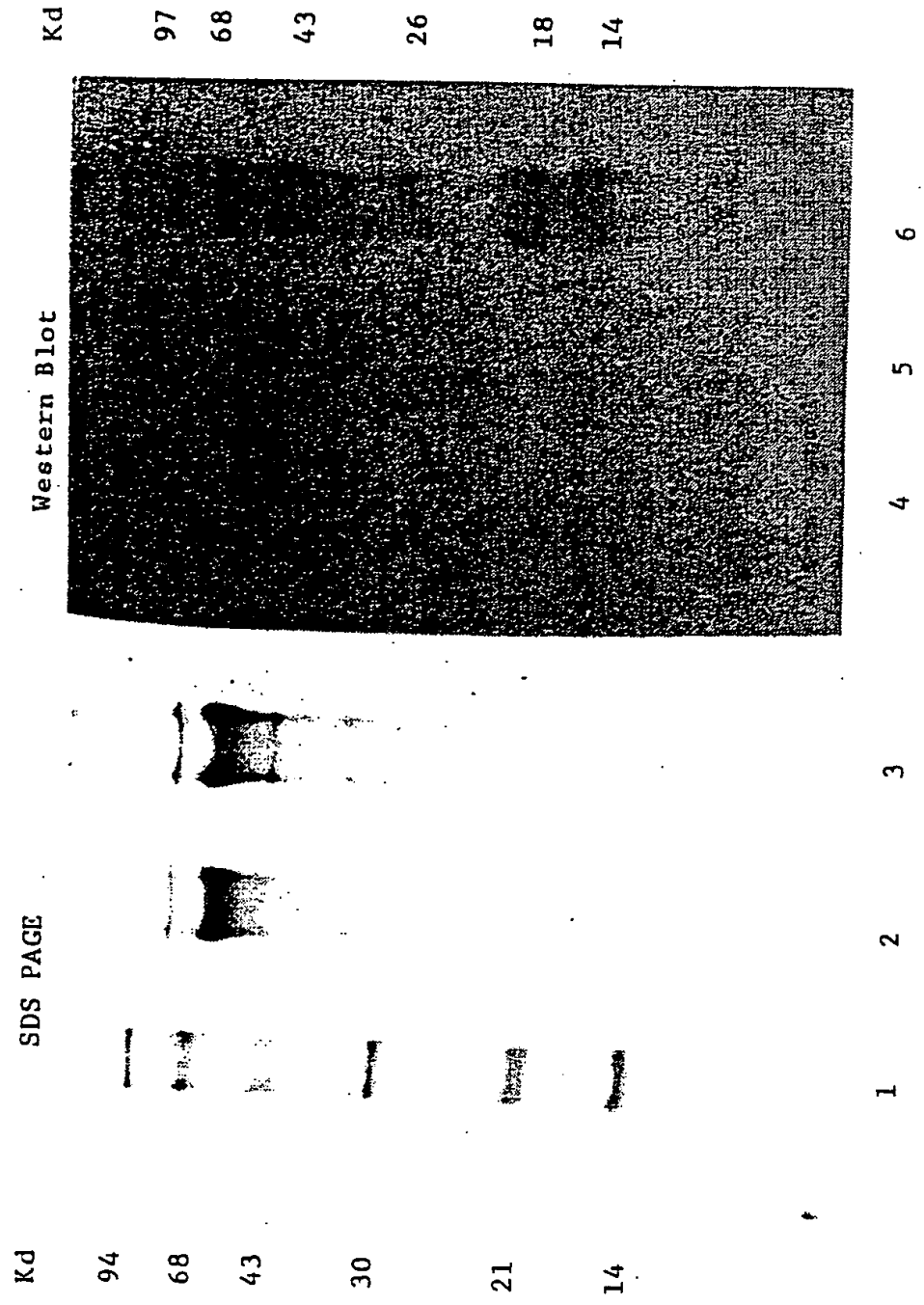
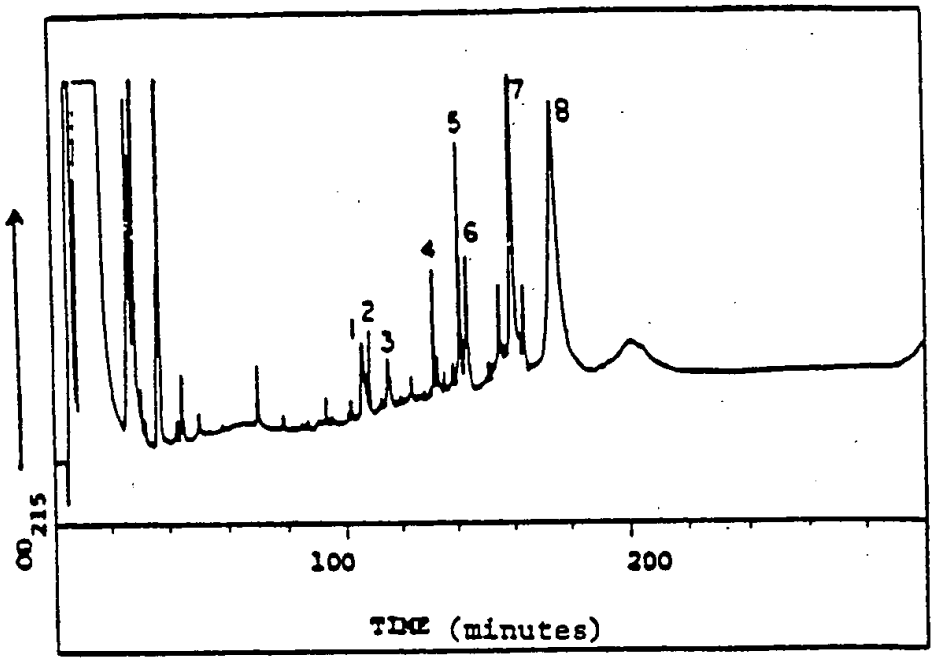

Kd
97
68
43
26
18
14
Western Blot
1
2
3
4
5
6
SDS PAGE
Kd
94
68
43
30
21
14

Fig. 23

A.


1
2
3
4
5
6
7
8
OD215
100
200
TIME (minutes)

B.

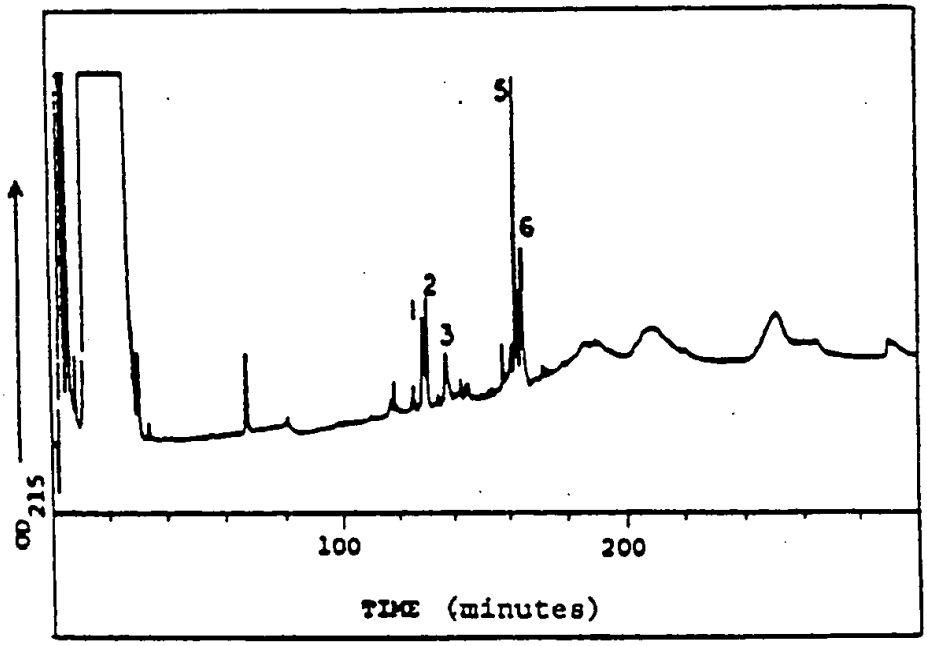

1
2
3
5
6
OD215
100
200
TIME (minutes)

Fig. 24

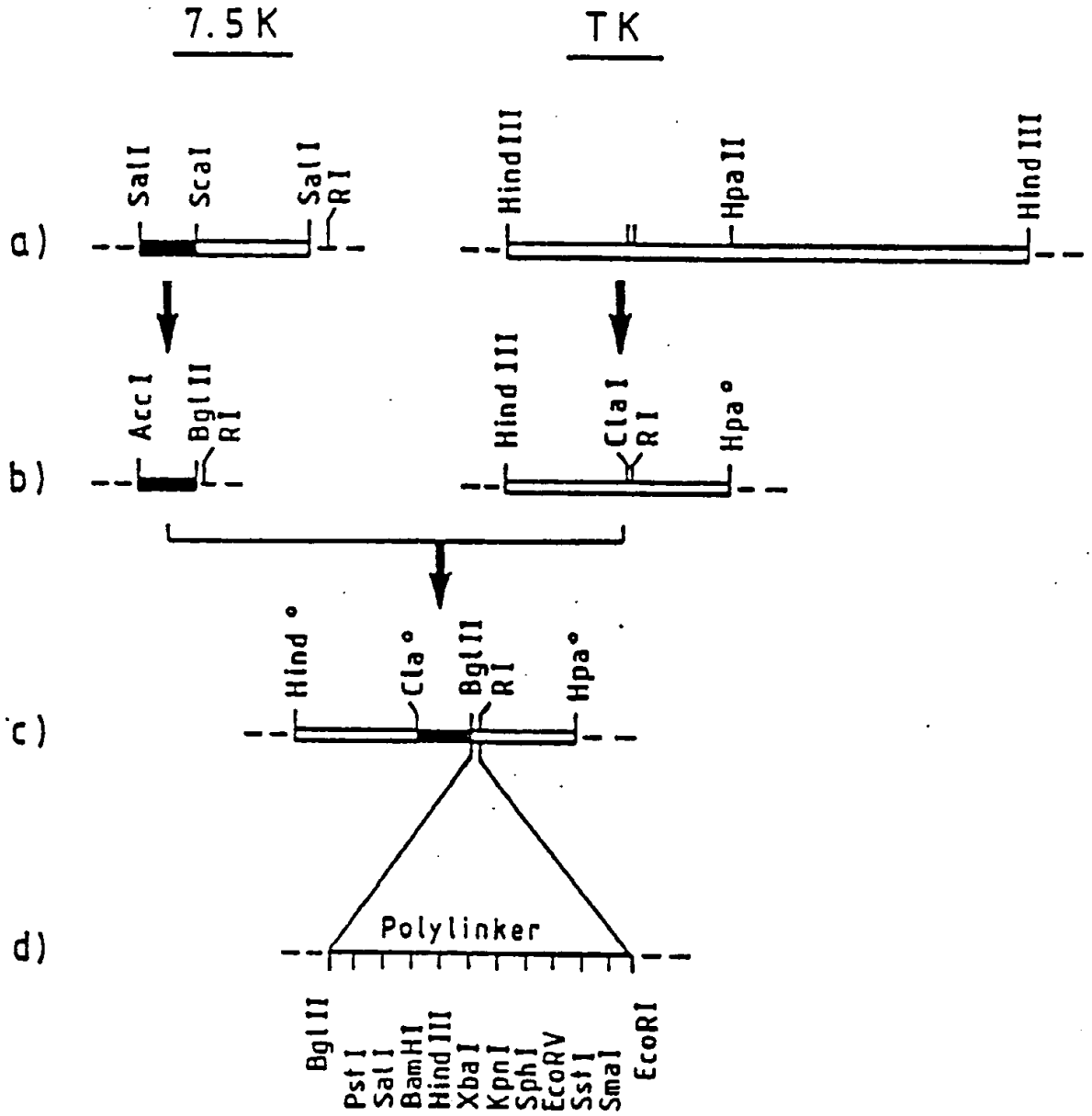

7.5 K
TK
a)
Sal I
Sca I
Sal I
R I
Hind III
Hpa II
Hind III
b)
Acc I
Bgl II
R I
Hind III
Cla I
R I
Hpa °
c)
Hind °
Cla °
Bgl II
R I
Hpa °
d)
Polylinker
Bgl II
Pst I
Sal I
BamHI
Hind III
Xba I
Kpn I
Sph I
EcoRV
Sst I
Sma I
EcoRI

Fig. 25

A

```
                    5                   10                  15
Met Arg Trp Glu Phe Pro Thr Ser Arg Glu Ala Pro Gly Ala Ser ....
```

B

```
                    5                   10                  15
Met Lys Ile Ile Phe Phe Leu Cys Ser Phe Leu Phe Phe Ile Ile
Asn Thr Gln Cys:Val Thr His Glu Ser Tyr Gln Glu Leu Val Lys
Lys Leu Glu Ala Ser Ser Arg Gly Thr Ala Cys Asp Ile Glu Leu
Ser Arg Glu Phe Pro Thr Ser Arg Glu Ala Pro Gly Ala Ser ....
```

European Patent Office

EUROPEAN SEARCH REPORT

Application number

DOCUMENTS CONSIDERED TO BE RELEVANT

EP 89110056.2

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
P,A	WO - A1 - 88/06 629 (SYNERGEN INCORPORATED) * Claims 1,3,7 *	1,22	C 12 N 15/00 C 12 P 21/00 C 07 K 13/00 C 07 H 21/04
A	EP - A2 - 0 135 712 (AMERICAN CYANAMID COMPANY) * Abstract; claim 1 *	1,24, 26	C 12 N 1/20 A 61 K 39/012
A	EP - A2 - 0 135 073 (AMERICAN CYANAMID COMPANY) * Abstract; claim 1 *	1,24, 26	
A	EP - A2 - 0 241 139 (MERCK & CO. INC.) * Claims 7,12-14 *	1,24, 26	
D,A	US - A - 4 650 676 (SCHENKEL et al.) * Claims *	1,24, 26	
A	EP - A2 - 0 231 537 (SOLVAY & CIE) * Claims *	1,6, 11,16, 18,19, 21,22, 24,26	TECHNICAL FIELDS SEARCHED (Int. Cl.4) C 12 N C 12 P C 07 K C 07 H A 61 K
A	EP - A2 - 0 164 176 (SOLVAY & CIE) * Claims *	1,6, 11,16, 18,19, 21,22, 24,26	
A	WO - A1 - 86/00 528 (GENEX CORPORATION) * Claims 1-3,14,31,41,42 *	1,11, 16	

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
VIENNA	22-08-1989	WOLF

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

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